**INTRODUCTION**

Organ formation is a highly regulated process during which groups of cells coordinately reorganize into specific three-dimensional forms. Cell migration is an essential aspect of organ formation and involves not only cell movement, but movement in a specific direction at the correct time. Formation of the highly elaborate branched network of the *Drosophila* tracheal system is driven by cell shape changes, cell rearrangements and directed cell migration, and provides an excellent system in which to study these events (Manning and Krasnow, 1993; Samakovlis et al., 1996). The trachea forms from ten bilaterally paired placodes of approximately 80 cells each located along the ventrolateral surface of the embryo. Tracheal cells invaginate from these ectodermal placodes to form individual cellular sacs, from which the primary branches arise. Directed migration of the five primary branches, plus subsequent branching and fusion, results in a complex tubular network which supplies all larval tissues with oxygen.

Genetic analysis of mutations affecting tracheal formation has revealed the roles of many genes (reviewed by Affolter and Shilo, 2000). The bHLH-PAS transcription factor encoded by *tracheless* (*trh*) is involved in the initial invagination step; in *trh* mutants all tracheal precursor cells remain at the embryo surface. The POU-domain transcription factor CF1α, encoded by *ventral veinless/driftier* (*vvl/dfr*), is also required early in tracheogenesis. TRH and VVL regulate known target genes necessary for the internalization of the primordia and subsequent branching events (see also figure 5 in Boube et al., 2000). Once internalized, the Fibroblast growth factor (FGF) homologue Branchless (BNL) and its receptor Breathless (BTL) are essential for primary branch formation; mutations affecting either molecule result in a fully internalized, but unelaborated sac of tracheal cells (see Fig. 1). The BNL signal is expressed in target tissues towards which tracheal cells normally migrate, and BTL is expressed in tracheal cells. Transduction of the BNL signal through BTL is thought to guide migration towards BNL sources.

In addition to the spatial guidance cues provided by BNL/BTL activity, at least three other signaling pathways have been implicated in primary branch formation: Decapentaplegic (DPP), Epidermal growth factor receptor (EGFR), and Wingless/WNT (see Fig. 1). DPP signaling is required to form the three branches that migrate along the dorsoventral axis: the dorsal branch (DB), the lateral trunk (LT), and the ganglionic branch (GB; Llimargas and Casanova, 1997; Vincent et al., 1997). DPP signaling in the dorsoventral branches activates expression of *knirps* (*kni*; Vincent et al., 1997), which encodes a transcription factor that mediates dorsoventral migration (Chen et al., 1998). EGFR signaling has an early role in invagination (Llimargas and Casanova, 1999) and is also reported to be involved in the migration of the dorsal...
Fig. 1. Multiple signaling pathways are required for tracheal branching. The top half of the figure depicts tracheal placodes (blue) at embryonic stage 10 and the sources of activation (magenta) of four signaling pathways known to function during tracheogenesis (EGFR, FGF, DPP, WG). The lower portion of the figure depicts tracheal metameres at stage 13/14. By this stage, wild-type trachea have formed the five primary branches: dorsal branch (DB), dorsal trunk anterior and posterior (DTa, DTp), visceral branch (VB), lateral trunk (LT), and ganglionic branch (GB). TC, transverse connective. Defects in the components of the pathways are schematized (representative genotypes of the mutants are noted below each metamere). White circles represent absence of appropriately migrating tracheal cells in mutant metameres; blue circles represent the observed positions of the tracheal cells. EGFR signaling is initiated by a localized source of RHO within the placode (magenta; Bier et al., 1990). In the absence of EGFR signaling, many cells fail to invaginate and remain clustered on the surface of the embryo, and cells are missing from every branch (see also Fig. 3G-H'). The BNL/FGF ligand is expressed in patches outside the trachea (magenta) and signals to the BTL/FGFR, which is expressed in the tracheal placode. In the absence of FGF signaling, all branches fail to migrate. The DPP ligand has localized sources dorsal and ventral to the placode (magenta) and signals through the receptors TKV (expressed in the placode) and PUT (expressed ubiquitously; Affolter et al., 1994; Ruberte et al., 1995). kni expression is DPP-dependent in the DB, LT, and GB. In the absence of DPP signaling, kni expression is lost in the DB, LT and GB, and these branches do not form. WG is expressed adjacent to the tracheal placodes (magenta) and signals to the tracheal placode to direct the transcriptional activities of ARM and dTCF. One downstream target of WG signaling is sal. In the absence of WG signaling, sal expression is lost, and DT cells fail to migrate away from the transverse connective (TC). It should be noted that other WNT molecules may be required to activate signaling through ARM/dTCF since arm and dTCF mutant phenotypes are stronger than the wg phenotype alone (Llimargas, 2000). In embryos lacking rib function, no DT is formed and the LT and GB are stunted in their migration. Stages are according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985).

trunk (DT) and visceral branch (VB; Llimargas and Casanova 1997; Wappner et al., 1997). Also, WG/WNT signaling is required for DT formation (Chihara and Hayashi, 2000; Llimargas, 2000). These pathways play positive, instructive roles in the formation of specific branches, and also have antagonistic activities. For example, the spalt (sal) gene is initially expressed throughout the dorsal half of the tracheal placode and is subsequently restricted to the DT cells (Kuhnlein and Schuh, 1996). Spatial regulation of sal expression is achieved by EGFR and WG/WNT signaling, which activate sal expression (Wappner et al., 1997; Chihara and Hayashi, 2000; Llimargas, 2000), and by DPP signaling in the DB cells, which activates KNI, a direct transcriptional repressor of sal (Chen et al., 1998). Expression of sal in the DT is necessary for DT formation (Kuhnlein and Schuh, 1996), whereas loss of sal expression in the DB is necessary for DB formation (Chen et al., 1998).

Although these four signaling pathways (FGF, DPP, EGFR and WG/WNT) are required for normal tracheal development, it is not yet known how information from these pathways is integrated to promote the subcellular changes necessary for directed cell migration. Clearly, the identification and characterization of new mutations affecting tracheal development will reveal many of the events that underlie normal tracheogenesis and how information from signaling pathways is integrated and implemented.

Here, we describe a role for the ribbon (rib) gene in the directed migration of the tracheal DT cells. rib was first identified in the large-scale EMS mutagenesis screen for defects in cuticle structure (Nüsslein-Volhard et al., 1984). Subsequently, Jack and colleagues identified a role for rib in Malpighian tubule development, and described cell shape defects in several ectodermally derived tissues (Jack and Myette, 1997; Blake et al., 1998; Blake et al., 1999). We initially identified rib as a candidate mutant for a gene that was expressed in the trachea under the control of TRH. Although we proved that rib does not correspond to that nearby gene, we were intrigued by the phenotypes of rib mutant embryos. In this paper, we report defects in the early trachea and salivary glands of rib mutants and show that these organs fail to complete the directed movements needed to give rise to their final shapes. In the tracheal DT, loss of rib function most closely resembles loss of WG/WNT signaling, suggesting that rib may link this or other signaling pathways to the cellular changes necessary to undergo directed migration along the anteroposterior axis of the embryo. We also report the cloning of the rib gene and show that it encodes a novel BTB/POZ protein expressed widely during Drosophila embryogenesis.
rub and directed cell migration

**MATERIALS AND METHODS**

**Fly strains**

The wild-type fly strains used were Canton S (chromosomes) or Oregon R (embryos). The following strains were also used and are described in FlyBase (wwwflybasebioindianaedu): Df2RIP34, Df2RIP7, Df2RG9, Df2RGC81, cord3, rho-AS8, rib1, rib2, sal1, tkv, wbl, wbl, wbl, wbl, and zip. Two independent wbl genomic transgenes were tested for rescue (Konsolaki and Schupbach, 1998). The Gal4/UAS system (Brand and Perrimon, 1993), using the transgenes UAS-sal (Kuhlnein and Schuh, 1996) and btl-Gal4 (Shiga et al., 1996), was used to express sal-TSE-lacZ reports the tracheal expression of sal (Kuhlnein and Schuh, 1996). rho-lacZ R1.1 is an insertion in the rho gene that expresses lacZ in the same pattern as the endogenous rho transcript (Wapper et al., 1997). The Ep(2)2445 line (Rorth et al., 1998) contains a single P-element insertion at position 268,109 in Celera Genomics contig AE003796. A standard excision mutagenesis (Df,2R) was performed as described previously (Lehmann and Tautz, 1994), using the following cDNAs as templates: bnl (S. Hayashi), btl (D. Montell), dpp (W. Gelbart), rib (LD16058, Research Genetics), and sal (LD17463, Research Genetics). In situ hybridized embryos were mounted on slides in 70% glycerol to limit diffusion of the alkaline phosphatase reaction products. Homozygous mutant embryos were identified by morphological criteria, by the lack of β-gal staining, or by the lack of antisense lacZ hybridization. Staining with antibodies to β-gal, or lacZ hybridization, detects embryos carrying a balancer chromosome with a lacZ insert, specifically CyO-flc-lacZ (CFL) or TM3-Ubx-lacZ (TUL). Embryos were visualized by Nomarski optics using a Zeiss Axiohot microscope. Ektar 25 or 100 print film (Kodak) was used for photography.

**Cuticle preparations**

Cuticles were prepared as described previously (Andrew et al., 1994), examined using both phase-contrast and dark-field optics, and photographed with TMAX 100 print film (Kodak).

**Molecular analysis**

BDGP cDNA clones (see Table 2) that mapped to unordered BACs in the 56C region were obtained from Research Genetics, sequenced, and grouped into genes. The genes were oriented relative to Celera Genomics clone AC020290, which was subsequently replaced by AE003797 and AE003796. The positions of these genes were mapped relative to local deficiency breakpoints by in situ hybridization to salivary gland polytene chromosomes, and was carried out according to procedures previously described (Pardue et al., 1994) using the Vectastain kit (Vector Laboratories) for HRP signal detection, omitting the RNase treatment and acetylation steps.

The prediction programs Gene Finder (http://dotimgenbctmc.edu:9331/gene-finder/gf.html) and GENSCAN (Burge and Karlin, 1997) were used to predict genes in the rib region. Predictions from these programs were compared with the sequences from the BDGP cDNA clones. In March 2000, annotated sequences for the entire region was released (Adams et al., 2000), which fully supported our molecular analysis.

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*All cDNAs that were sequenced in their entirety on both strands as part of this analysis.*
The RIB protein was analyzed by InterPro (http://www.ebi.ac.uk/interpro), Pfam (http://pfam.wustl.edu), PROSITE (http://expasy.cbr.nrc.ca/prosite/), and PSORT II (http://psort.nibb.ac.jp/form2.html). Homology searches were performed using BLAST (http://www.ncbi.nlm.nih.gov). Alignments were generated by CLUSTALX (Thompson et al., 1997) and illustrated using MacBoxshade (http://www.netaxs.com/jayfar/mops.html).

Sequence analysis of candidate ORFs in rib mutants

Two sources of template were used to amplify small overlapping regions of candidate gene ORFs by PCR: single embryos (rib/rib; selected for a lack of GFP expression from a Kruppel-GFP transgene on the CyO balancer chromosome (Casso et al., 1999) or genomic DNA isolated from heterozygous adult flies (rib/CFL). PCR with single embryos as template was performed as described previously (Franc et al., 1999). PCR products were sequenced and analyzed for allelic differences. All changes were verified by sequencing the corresponding regions on the opposite strand. DNA sequencing was performed at the Johns Hopkins University Core DNA Analysis Facility.

RESULTS

rib is required for specific directed cell migration in the trachea

rib mutants showed gross abnormalities in tracheal morphology at late stages (Fig. 2A,B), consistent with the phenotypes previously reported (Jack and Myette, 1997). To determine when defects were first apparent and if specific branches were affected, we examined rib mutant embryos using a panel of tracheal markers. Expression of three of the earliest expressed transcription factors, TRH, VVL/DFR and KNI, was completely normal at the placode stage, as was expression of two of their targets, btl and rhomboid (rho; Fig. 2C,D, and data not shown). These results indicated that specification of the tracheal primordia was unaffected in rib mutants. Moreover, there were no overt differences in the shape or size of tracheal placodes in rib mutants, and the invagination of tracheal cells was initiated with similar timing and positioning as in wild type. Differences were first apparent during stage 12, when outgrowth of branches was either absent or delayed (Fig. 2E,F). By stage 14, rib tracheae were clearly defective in a subset of branches (Fig. 2G,H). In most segments, the dorsal trunk was completely absent, and cells were clustered at the position within the tracheal sac from which DT cells normally migrate. In a few metameres, the cluster of DT cells in the trachea had fewer cells, and the corresponding DB contained additional cells. The LT and GB were also defective; both branches were stunted, and often the LT was completely absent. Unlike the DTs, which only rarely migrated, migrating GBs were frequently observed.

![Fig. 2. rib mutants have defects in tracheal development. Embryos in the left column are wild-type; embryos in the right column are rib homozygotes. A-H, K, and L are lateral views; I and J are dorsal views. 2A12 or anti-CRB was used to visualize the tracheal lumen, and anti-TRH to visualize nuclear TRH in all tracheal cells. The tracheal network is abnormal in rib mutants (B). By late stage 12, many of the tracheal branches in rib mutants have not migrated and lumen size is expanded (F), as compared with wild type (E). Insets in E,F,G,H are of metamere 4 (black arrow). (G,H) At stage 14, the DT (white arrow) is clearly absent in rib mutants (H) and the LT (black arrowhead) and GB (arrow) are stunted. The VB (white arrowhead) is visible out of the plane of focus (H). In late stage rib embryos (J), VBs reach the gut and perform terminal branching (arrows), as in wild type (I). (K,L) In embryos carrying UAS-rib and the tracheal driver btl-Gal4, tracheal phenotypes are rescued. (K) The lumina are less dilated, the two ventral branches are less stunted (black arrow and arrowhead), and DBs are not present (white arrow). (L) Rescue of DT formation is obvious by late stages (arrow). On average, seven of the nine DT fragments form in these rescued embryos. Wild-type genotypes are rib/CFL, which were also stained with anti-βgal (brown staining in C,E,G), or Oregon R (A,I). No differences were observed in the phenotypes of embryos carrying one versus two wild-type alleles of rib.](image-url)
at later stages, although they were often misrouted (data not shown). Migration of the other two primary branches (DB and VB) was generally unaffected, except for occasional extra DB cells (Fig. 2). The lumina of all branches of rib tracheae appeared more dilated than in wild type. It is possible that lumenal dilation is related to the early slowed outgrowth and stunting, either as a cause or effect. Alternatively, the two phenotypes could be independent. Both the lumen size and the migration defects in rib mutants are rescued by tissue-specific rescue with a rib transgene (Fig. 2K,L, see below), which demonstrates that rib function is required in tracheal cells.

rib and known signaling pathways in the trachea

To learn how rib functions with known signaling pathways to promote tracheal formation, we compared the tracheal defects in rib mutants with those in embryos in which the FGF, DPP, EGFR, or WG/WNT pathways were disrupted by following the expression of pathway-dependent tracheal markers. In the trachea, FGFR/BTL signaling is regulated by the spatially restricted expression of FGF/BNL (Fig. 1; Sutherland et al., 1996). To determine if rib defects were due to changes in btl or bnl expression, we examined RNA accumulation in rib mutants. Both btl and bnl RNA expression patterns were normal in rib mutants, except for a slightly prolonged expression of btl in the transverse connective (TC; Fig. 3A-D). Additionally, FGF-dependent MAPK activation at the tracheal pit stage (Gabay et al., 1997) was normal in rib mutants, as detected by staining with an antibody to the diphosphorylated, activated form of ERK (dpERK; data not shown). These findings reveal that rib does not function upstream of FGF signaling and that rib acts either downstream of or in parallel to FGF-dependent dpERK activation.

rib mutants exhibited only a subset of defects observed in FGF signaling mutants; thus, if rib functions downstream of FGF signaling, it must do so in a spatially or temporally restricted manner.

DPP signaling is required for the DB, LT and GB, where it activates KNI expression which, at least in part, mediates the dorsoventral migration of these branches (Vincent et al., 1997; Chen et al., 1998). Since the incomplete migrations of the LT and GB in rib mutants could be caused by reduced DPP signaling, we asked whether dpp expression or KNI maintenance in the LT or GB was affected in rib mutants. dpp RNA was detected in the wild-type expression pattern, both dorsally and ventrally to the tracheal placode (data not shown).
As in wild-type embryos, KNI accumulation in rib mutants was detected in the early rib tracheal placode (data not shown) and was maintained in all branches of the trachea except the DT and TC, where it is normally lost (Fig. 3E,F). Thus, the stunted ventral branches in rib mutants are not caused by loss of KNI expression, and rib does not function upstream of DPP signaling. Moreover, if rib is required for DPP-dependent tracheal cell identity or migration, its requirement is limited to ventral cells (LT and GB) and is downstream of or parallel to activation of kni expression.

EGFR and WG/WNT signaling are both implicated in DT formation. Thus, rib could function with one or both of these pathways to promote DT migration. EGFR signaling was reported to specifically affect the formation of the DT and VB and to maintain expression of sal in the DT (Wappner et al., 1997). In embryos lacking EGFR signaling due to mutations in the receptor (DER/faint little ball), the ligand (spitz), or either of two upstream activators (Star and rho), many tracheal cells remain clustered near the tracheal pit (Wappner et al., 1997). Further analysis showed that not all placodal cells invaginate in rho mutants, leaving many cells on the surface of the embryo (Llimargas and Casanova 1999). Our analysis of rho mutants revealed that more DT cells and VB cells undergo normal primary branch migration than previously reported (Fig. 3G-J; Wappner et al., 1997). We also observed a significant loss of DB formation, and in most rho embryos, all branches contained fewer cells (Fig. 3G-H'). Moreover, we detected sal RNA expression in the dorsal region of the pits during stages 11-14 in rho mutants, although levels were not quite as high as in wild type (Fig. 3I,J). This result is in contrast to previous reports that sal is only expressed in the limited DT fragments that form between adjacent metameres in rho embryos (Wappner et al., 1997). Our findings support one of two models proposed by Llimargas and Cassanova (Llimargas and Cassanova, 1999) in which EGFR signaling is required for tracheal cell invagination, and defects in branch migration are an indirect consequence of having fewer cells at the appropriate position to receive and respond to spatial cues required for appropriate migration (such as WG signals to DT cells, see below). In contrast to defects caused by loss of EGFR signaling, mutations in rib affected DT migration more directly: all tracheal cells in rib mutants appeared to invaginate from the ectoderm, and DT cells were usually observed in a cluster at the TC below the DB cells. Additionally, expression of rho, which is involved in the spatial activation of EGFR, and EGFR-dependent dP-ERK were normal in rib mutants (data not shown), further indicating that rib acts independently of EGFR signaling.

Loss of WG/WNT signaling causes DT defects similar to those in rib mutants: the DT is absent and ‘pre-DT’ cells are clustered below the DB (Chihara and Hayashi, 2000; Llimargas, 2000). The only known early target of WG/WNT signaling in the trachea is the sal gene (Chihara and Hayashi, 2000; Llimargas, 2000), which is also required for proper DT migration (Kuhnlein and Schuh, 1996). In rib mutants, the spatial and temporal patterns of both sal RNA and SAL protein accumulation were unaffected, although levels appeared slightly reduced compared to those in wild-type embryos (Fig. 4A-D; data not shown). Since expression of sal in all tracheal cells (btl-Gal4/UAS-sal) did not increase DT cell migration (Fig. 4E,F), the slight reduction in sal expression

![Fig. 4. rib functions downstream of, or in parallel to, WG signaling and is independent of sal. All views are lateral. Early stage 12 wild-type (A) and rib (B) embryos hybridized with an antisense sal RNA probe show sal accumulation in dorsal tracheal cells (arrow). Wild-type (C) and rib (D) embryos carrying the sal-TSE-lacZ reporter construct stained with anti-β-gal, which detects expression in the dorsal tracheal cells (arrow), and with anti-CRB to visualize the trachea. Slightly lower levels of sal-β-gal are detected in rib mutants. Anti-CRB staining of a rib embryo carrying both UAS-sal and btl-Gal4 transgenes (F) reveals that DT migration (arrow) is not rescued with increased sal expression compared with rib alone (E). The DT (arrow) in rib mutants fails to migrate (G), whereas the DB (arrowhead), LT, and GB fail to form in tkv (I) mutants (H). Embryos doubly mutant for rib and tkv (I,J) form only the TC (arrow in I) and VB (arrow in J).


does not contribute to the rib phenotype. These experiments demonstrate that rib functions in DT cells downstream of or parallel to WG/WNT signaling and independently of sal.

In embryos doubly mutant for WG/WNT and DPP signaling, only VB cells migrate (Llimargas, 2000). Similarly, in embryos doubly mutant for rib and thick veins (tkv), which encodes one of the receptors essential for DPP signaling (Affolter et al., 1994), only VB cells migrated (Fig. 4G-J), a phenotype identical to that of embryos doubly mutant for tkv and armadillo (arm; Llimargas, 2000), which encodes an essential component of WG/WNT signaling (Peifer et al., 1993). Thus, like WG/WNT signaling, rib plays an instructive role in DT formation.

In sal mutants, DT cells migrate dorsally instead of forming the DT (Kuhnlein and Schuh, 1996), whereas in WG/WNT signaling mutants, DT cells are stalled at the TC. Thus, WG signaling must regulate other genes, in addition to sal, that control migration. Given that rib appears to phenocopy the loss of WG signaling in the DT, and that rib functions downstream of or in parallel to WG/WNT signaling, rib itself might be a target of WG/WNT signaling. rib RNA is expressed throughout the epidermis and is not obviously upregulated in the trachea (see below). Thus it is unlikely that rib is transcriptionally controlled by WG/WNT or other signaling pathways.

**rib function in the epidermis**

rib mutants fail to complete dorsal closure (Nüsslein-Volhard et al., 1984; Jack and Myette, 1997), the process by which the cells of the lateral epidermis move dorsally to encompass the amnioserosa and seal the dorsal surface of the embryo (reviewed by Noselli and Agnes, 1999). The Jun N-terminal kinase (JNK) signaling pathway (reviewed by Noselli and Agnes 1999) and the WG signaling pathway (McEwen et al., 2000) are required for dorsal closure. Both pathways are necessary for the characteristic cell shape changes in the leading edge cells and the transcriptional activation of dpp. As reported by Blake et al., rib mutants also lack the characteristic elongation of the cells at the leading edge, and at late stages, these cells are large and misshaped (Blake et al., 1998).

To determine whether the dorsal closure defects in rib mutants are related to defects in JNK or WG signaling, we analyzed the dorsal cuticle of larvae carrying different allelic combinations of rib mutations. In the allelic combinations that could be scored (i.e., those in which sufficient cuticle was produced), approximately two-thirds of the larvae had a large dorsal hole, and one-third had a small anterior dorsal hole with a puckering of the remaining dorsal cuticle (Fig. 5A-C). This range of phenotypes is similar to the defects in larvae with loss-of-function mutations in either JNK or WG pathway components. We also investigated whether dpp expression was maintained in the leading edge cells of rib mutants. Unlike mutations in either JNK or WG signaling components, in which dpp expression is absent in leading edge cells dpp expression was observed at high levels in the leading edge cells in rib mutants (Fig. 5D,E). At late stages, dpp expression was often observed in lateral patches. This apparent increase of dpp expression in rib mutants could be due to increased numbers of cells expressing dpp, increased size of leading edge cells and/or loss of cell cohesion (which could cause cells to

![Fig. 5. rib mutants fail to complete dorsal closure and have ventral cuticle patterning defects. Dark-field images of the lateral cuticle of wild type (A) and rib mutant larvae (B,C) photographed at the same magnification. Of the mutant larvae with scorable cuticles, small anterior dorsal holes and puckering of the dorsal epidermis occur in 36% of rib mutants (B) and large dorsal holes occur in 64% of rib mutants (C; arrows indicate extent of dorsal opening). Lateral views of dpp RNA expression in the leading edge cells of the lateral epidermis (large arrow) and the midgut (arrowhead) of rib/CFL (D) and rib mutant (E) embryos are shown. In rib mutants, dpp staining at the leading edge is more disorganized and in some regions extends into the lateral epidermis (small arrows in E). This apparent increase may be due to an increase in the number of cells expressing dpp or the morphology of these cells and the leading edge at late stages. (F-J) Representative phase contrast images of ventral cuticles of first instar larvae of an allelic series of rib mutations. Anterior (A) is oriented up, and larvae were photographed at the same magnification. There is a prominent narrowing of the lateral extent of denticle belts relative to the ventral surface of the larva, increasing with allele severity. Loss of denticle diversity also increases with the allelic series. The most severe phenotype (rib/rib) is shown in J and is equivalent to rib/Df(2R)P34 (not shown); in such embryos, only a few similarly shaped denticles form.](image-url)
collapse or remain in more ventral positions). In any case, this experiment reveals that, as in the trachea, rib is not an upstream activator of JNK or WG signaling and that JNK- and WG-dependent activation of dpp is not mediated by rib. Mutations in rib caused defects at an earlier step in dorsal closure than dpp mutations: the leading edge cells fail to change shape in rib mutants, whereas DPP signaling is required for cell shape changes and movement of the ectodermal cells just ventral to those at the leading edge (Riesgo-Escovar and Hafen, 1997). Thus, if rib functions downstream of the JNK or WG pathway to mediate dorsal closure, it must be acting in parallel to dpp activation.

Patterning in the ventral cuticle is also impaired in rib larvae, which exhibit both a narrowing of the lateral extent of denticle belts and a fusion of belts at the midline (Fig. 5F-J; Nüsslein-Volhard et al., 1984). At a gross level, these phenotypes are similar to those described for the EGFR signaling mutants, rho and spitz (Raz and Shilo, 1993). We examined the cuticle phenotypes of different allelic combinations of rib mutations, scoring both the lateral extent of denticle belts and denticle diversity. The lateral extent of rib denticle belts was narrowed to 37%, 46% and 70-100% of the wild-type width, consistent with transcriptional activation of early target genes. Thus, if rib functions downstream of or parallel to these pathways to promote cellular changes, it must be acting in parallel to dpp activation.

The loss of denticle diversity in rib mutants also corresponded to the above allelic series. The least affected cuticles had the most diversity of denticle types (rib<sup>2</sup>/rib<sup>2</sup>), Fig. 5G), whereas more severely affected cuticles had only one or two denticle types (rib<sup>2</sup>/rib<sup>1</sup> and rib<sup>2</sup>/DfD; Fig. 5H,J), and the most severely affected cuticles had very few faint denticles which appeared to be of a single type (rib<sup>1</sup>/rib<sup>1</sup> and rib<sup>1</sup>/Df; Fig. 5F-J). The denticle belts of rib larvae with a single denticle type looked notably similar to larvae simultaneously lacking the late activities of WG and EGFR signaling, in which all denticles are type 5 (w<sup>g6</sup>, UAS-DN-DER, arm. Gal4; Szuts et al., 1997). Unlike WG/EGFR-deficient larvae, however, not all of the denticles in rib mutants were oriented posteriorly. Overall, the dorsal and ventral cuticle phenotypes, together with the tracheal defects, suggest that rib may play a role in maintaining organ shape once the salivary gland has formed. Expressing a rib transgene specifically in the secretory cells of rib mutants (see below) restored both directed migration and lumen size (Fig. 2I,J). Thus, rib function is required in secretory cells to control migration and organ shape.

The salivary duct also failed to undergo proper morphogenesis in rib mutants. Two duct markers, TRH protein and btl RNA, were detected in a normal pattern in the duct primordia of rib mutants (data not shown). In contrast, duct cells stained poorly for the Dead ringer (DRI) protein, which is normally expressed robustly by stage 13; only diffuse low levels of DRI expression were detected in rib mutants prior to stage 15. In late stage rib mutant embryos, we observed either no tubes or rudimentary individual tubes connected to the secretory glands; these semi-tubular structures did not elongate and never elaborated into a normal duct (Fig. 6L,M). In embryos expressing a rib transgene in secretory cells of rib mutants (see below), duct formation was restored (Fig. 6N). This result indicates that rib duct defects are indirect and suggests that duct formation requires proper secretory cell morphogenesis. While both salivary gland structures are normally formed in rib mutants, there is a specific requirement for rib in the secretory cells for their posterior migration, similar to the requirement for rib in the tracheal DT cells for their anteroposterior migration.

**Directed migration of the salivary gland is defective in rib mutants**

Signaling pathways controlling cell migration in the embryonic salivary gland have not yet been identified. Nonetheless, the salivary gland, like the tracheal system, invaginates through a stereotypical process involving directed cell migration (Fig. 6; reviewed by Myat et al., 2000) The salivary glands form from two paired primordia that arise from the ventral ectoderm of parasegment two. Through changes in cell shape and migration, the primordia are internalized and ultimately give rise to two cell types: secretory and duct. The secretory cells are the first to invaginate and proceed in an ordered, sequential manner beginning with the cells in the dorsal posterior region of the primordium (Myat and Andrew, 2000). The secretory cells move dorsally into the embryo, then turn and migrate posteriorly until the distal half of the gland reaches the level of the third thoracic segment. After the movements of head involution, the salivary glands lie closer to the anterior end of the embry and are oriented along the anteroposterior axis. Concomitant with later secretory cell migrations, the duct cells undergo a complex set of morphogenetic movements to create a tubular structure. This tube starts at the larval mouth and then branches to connect to the two secretory glands (Fig. 6K; Kuo et al., 1996).

Although salivary glands were reported to be abnormal in late stage rib mutant embryos, earlier stages were not analyzed (Jack and Myette, 1997). As with the tracheal primordia, the secretory gland primordia in rib mutants were indistinguishable from those in wild-type embryos, and the initial invagination proceeded normally (Fig. 6A,B); however, rib secretory cells did not migrate past the point at which wild-type cells turn and migrate posteriorly (Fig. 6C-F). Thus, the secretory cells in rib mutants never reach their final destination. At late stages, the lumina of the salivary glands were greatly enlarged compared to wild-type glands (Fig. 6G,H), suggesting that rib may also play a role in maintaining organ shape once the salivary gland has formed. Expressing a rib transgene specifically in the secretory cells of rib mutants (see below) restored both directed migration and lumen size (Fig. 2I,J). Thus, rib function is required in secretory cells to control migration and organ shape.

**Identifying the rib transcripts**

Our phenotypic analysis suggested that rib may respond to signals by activating changes required for directed cell movements during organogenesis. To understand the molecular mechanism by which rib functions, it was essential to identify and characterize the rib gene. rib maps to 2-88 (Tearle and Nüsslein-Volhard, 1987) and is uncovered by Df(2R)P34 (Blake et al., 1998). Complementation analysis with overlapping deficiencies revealed that rib function was also removed by Df(2R)GC8, but not by Df(2R)GC10, Df(2R)F7, or EP(2)2445<sup>5A</sup>, a small deficiency we generated using a nearby P element (Fig. 7; Table 3). Thus, rib maps distal to EP(2)2445<sup>5A</sup> and proximal to or spanning the distal breakpoint.
of \textit{Df(2R)P34}. By mapping deficiency breakpoints in combination with sequence information from the Berkeley Drosophila Genome Project (BDGP) and Celera Genomics (Adams et al., 2000), we identified three genes in this interval: \textit{windbeutel (wbl)} and two uncharacterized genes (Fig. 7B). Mutations in \textit{wbl} complemented \textit{rib} (Table 3), and a genomic \textit{wbl} transgene (Konsolaki and Schupbach, 1998) did not rescue \textit{rib} lethality in any allelic combination (data not shown). Based on the complementation data and on the observation that \textit{wbl} mutants did not exhibit embryonic defects similar to \textit{rib} (data not shown), we conclude that \textit{rib} is not allelic \textit{wbl}.

We examined the RNA expression patterns of the two remaining genes that map to the \textit{rib} interval (genes 4 and 5). Gene 4 was not detectably expressed in wild-type embryos as assayed by whole-mount in situ hybridization and northern analysis (data not shown). Since \textit{rib} function is required during embryogenesis, gene 4 is not likely to encode RIB. The remaining candidate, gene 5, was expressed in a complex and dynamic pattern in nearly all tissues during embryonic development, with higher levels in several tissues affected by \textit{rib} mutations including salivary gland, epidermis, and Malpighian tubules (Fig. 8). We also detected nucleotide (nt) changes in the corresponding open reading frame (ORF) in both \textit{rib} alleles (see below). Finally, by expressing gene 5 ORF in the tracheal system and salivary gland, we detected tissue-specific rescue of tracheal branch migration (Fig. 2K,L) and salivary gland posterior migration (Fig. 6I,J). This experiment conclusively demonstrated that gene 5 encodes RIB and that RIB function is required in tracheal and salivary gland secretory cells to control migration.

\textit{rib} encodes a novel BTB/POZ protein

\textit{rib} encodes a 1983 nt ORF corresponding to a 661-residue protein with an amino-(N) terminal BTB/POZ (Bric a brac, Tramtrack, Broad-Complex/Poxvirus, zinc finger) domain (Fig. 9). The BTB/POZ domain is an evolutionarily conserved...
domain that mediates homo- or heterodimerization with other BTB/POZ domains (Bardwell and Treisman, 1994; Chen et al., 1995) and is found in over 400 proteins. A short coiled-coil region in the carboxyl (C) terminus is predicted. RIB has four consensus nuclear localization signals (NLS), two of which are bipartite, and RIB is predicted to be nuclear by the PSORT program. Thus, RIB may function in the nucleus. RIB also contains consensus phosphorylation sites for a number of kinases that mediate the formation of tissues in which rib is required (e.g., MAPK). Consensus glycosylation, myristylation and amidation sites are also present in the rib ORF.

In the more severe allele rib1, we detected a single nucleotide change in the rib ORF that results in a nonsense codon after residue 282 (Fig. 9B). This mutation deletes the entire C-terminal half of the protein, and is likely to be null, consistent with phenotypic analysis. rib2 has a single base change that replaces arginine 58 with a histidine (R58H) in the BTB/POZ domain. A mutation in rib was also discovered on the zipper1 (zip1) chromosome (Blake et al., 1998). We confirmed that zip1 mutants fail to complement both rib alleles, sequenced the rib ORF on the zip1 chromosome, and found the identical nucleotide change that created the R58H mutation in rib2. This result is consistent with the phenotypic report that, when recombined off the zip1 chromosome, the rib1 allele behaves like rib2 and is not as severe as rib1 (Blake et al., 1998). It is not clear whether the zip1 chromosome recombined with rib2 at some point, or whether finding the identical residue substitution indicates the importance of R58 in RIB function.

All other detected base changes that resulted in residue substitutions were detected on all chromosomes and/or on the balancer chromosome, the balancer chromosome recombined at some point, or whether finding the identical residue substitution indicates the importance of R58 in RIB function. All other detected base changes that resulted in residue substitutions were detected on all rib chromosomes and/or on the balancer chromosome, suggesting that these other changes are polymorphisms not responsible for rib phenotypes.

**DISCUSSION**

rib is required for the formation of several embryonic tissues. In rib mutants, both tracheal cells and salivary gland secretory cells fail to complete characteristic migrations along the anteroposterior axis, indicating that rib is required for directed cell movements. Late in embryogenesis, once organs have...
formed, the salivary gland lumen becomes abnormally dilated, suggesting a role for rib in cell shape maintenance. In tracheal development, rib may function with WG/WNT and/or FGF signaling to direct the migration of the cells that give rise to the dorsal trunk. Incomplete dorsal closure and defective ventral cuticle patterning in rib mutants are also consistent with an interaction of rib with WG and/or MAPK signaling. Our phenotypic analysis of rib suggests that directed cell movements are regulated by the same components used in cell shape maintenance. Lastly, we show that the rib gene is expressed widely throughout development and encodes a novel protein with a BTB/POZ domain.

rho, rib and tracheal branch formation
We have made two observations that support a model proposing that the primary role for EGFR signaling is the invagination of tracheal primordia and that defects in branch migration may be an indirect result of reduced invagination (Llimargas and Casanova, 1999). (1) All branches contained fewer cells in rho mutants, hence no particular branch identity is lost. (2) sal expression in the dorsal tracheal cells of rho mutants during primary branch outgrowth is normal, suggesting that EGFR is not required to specify cell fate within the placode (at least as measured by expression of this DT-specific gene). Thus, EGFR signaling may only regulate invagination, which would position cells to receive subsequent signals specifying branch fate.

Analysis of WG signaling in tracheal branching (Chihara and Hayashi, 2000; Llimargas, 2000) suggests that cells are allocated to branches (cell allocation) independently from cell fate specification. (1) In WG signaling mutants the ‘pre-DT’ cells are positioned correctly, but fail to migrate away from the TC. (2) WG signaling mutants do not express sal, a DT-specific marker. Thus, the cells are allocated to the DT, but do not express DT markers or behave like DT cells. rib mutants, like WG/WNT signaling mutants, also failed to form the DT, and ‘pre-DT’ cells were stalled at the TC; however, unlike embryos lacking WG signaling, rib mutants expressed sal in DT cells. Thus, rib is not required for cell allocation or cell fate specification (as monitored by sal), but is only required for branch migration. In summary, these observations suggest that, at least for the tracheal DT, cell allocation is independent of cell fate specification, and cell fate can be further subdivided into branch identity (controlled by genes such as sal that specify branch features; Chen et al., 1998) and branch migration, which involves rib. We are currently limited by the number of markers available to assess specific tracheal cell fates and the independence of these processes. The identification of new branch-specific markers and mutations will allow us to further refine models for tracheal branching.

rib may function with multiple signaling pathways
The similarity of the tracheal DT phenotypes in rib mutants and WG signaling mutants raises the possibility that rib...
functions with WG signaling for migration of DT cells. sal is the only known early downstream target of WG/WNT signaling in the DT. Because the DT phenotype is more severe in embryos lacking WG/WNT signaling than in sal mutants, there must be additional downstream targets of WG signaling. Indeed, we can predict that these other genes control migration based on two findings. (1) DT cells are capable of migrating in sal mutants, but move in the wrong direction (dorsally; Kuhnlein and Schuh, 1996). (2) When both WG and DPP signaling are activated in wild-type embryos (activated arm and activated tkv in all tracheal cells), a complete longitudinal DT forms that does not express sal (Llimargas, 2000), suggesting that sal may be dispensable for anteroposterior migration in some cases. We have shown that loss of rib results in a DT phenotype identical to that observed in loss of WG/WNT signaling and that rib functions in parallel to WG/WNT-dependent sal expression. Together these results suggest that rib is working with WG/WNT signaling, either in parallel or potentially as a downstream target, to direct DT migration.

We hypothesize that rib may respond to signals from multiple pathways based on our analysis of the ventral cuticle phenotype. In rib mutants, the defects in ventral cuticle patterning appeared most similar to the phenotype reported for the combined loss of late WG signaling and EGFR signaling (Fig. 5; Szuts et al., 1997). In this tissue, rib could be integrating signaling from WG and EGFR. In several other tissues requiring rib function, WG signaling and signaling through a MAPK cascade are also required; however, in these cases, loss of either of the individual pathways results in phenotypes similar to those of rib mutants. For instance, rib is required for the cell shape changes in the leading edge cells during dorsal closure (Blake et al., 1998), a process that requires both WG signaling (reviewed by Noselli and Agnes 1999) and JNK signaling (McEwen et al., 2000). The second midgut constriction and the morphogenesis of the Malpighian
rib and directed cell migration 3013

rib may direct cell movements by regulating the cytoskeleton

rib is thought to be required for generating specialized cell shapes. For instance, during dorsal closure, leading edge cells of the lateral epidermis fail to elongate in rib mutants (Blake et al., 1998). rib mutants also show abnormal dilation of salivary gland lumina in late embryogenesis (this work; Jack and Myette, 1997), suggesting that either rib is also required at late stages to maintain organ shape or loss of early rib function indirectly causes the late luminal dilation. rib appears to control cell shapes by regulating the cytoskeleton. During dorsal closure, a band of actin and myosin forms at the dorsal margin of leading edge cells (Young et al., 1993). In rib embryos, the actin band is narrower and myosin heavy chain (MHC) is absent from leading edge cells (Blake et al., 1998). Thus, rib may be required for the localization or organization of cytoskeletal components. zip encodes a nonmuscle MHC and is required in many of the same tissues as rib; however, strong loss-of-function mutations in zip suppress the distended luminal phenotype of rib salivary glands, suggesting that rib does not positively regulate myosin activities (Blake et al., 1999). Instead, rib may repress myosin contraction or regulate the direction of contraction, perhaps by providing a balancing force to the direction of basal myosin contractions. Our studies reveal a role for rib in coordinating directed cell migration, a process that clearly involves actin/myosin dynamics. Thus, rib may modulate actin/myosin behavior for cell movement and cell shape during both tissue formation and tissue homeostasis. If rib is responding to signaling pathways, rib could be a critical factor linking signaling events to changes in the cytoskeleton.

The rib gene encodes a novel protein with two protein-protein interaction domains, an N-terminal BTB/POZ domain and a C-terminal coiled-coil region. The BTB/POZ domain mediates dimerization (Bardwell and Treisman, 1994; Chen et al., 1995), and BTB/POZ proteins often contain additional domains that define protein function and/or subcellular localization. Many BTB/POZ proteins contain multiple DNA binding zinc fingers and function as transcriptional regulators. For example, the Drosophila Tramtrack protein is required to represses transcription of pair-rule genes in early embryogenesis (Harrison and Travers, 1990). BTB/POZ domain proteins can also mediate cytoskeletal organization. For instance, the Drosophila Kelch protein, which oligomerizes via its BTB domain and binds actin through its kelch domains, is required to maintain cytoskeletal organization of ring canals during oogenesis (Robinson and Cooley, 1997). BTB/POZ proteins can also function outside the cell; the mammalian BTB/POZ protein Mac-2 binding protein (M2BP) localizes to the extracellular matrix (ECM) and forms multivalent ring structures proposed to be important for its interactions with collagens IV, V and VI, fibronectin, and other ECM proteins (Müller et al., 1999). One family of Arabidopsis BTB/POZ-containing proteins has a composition very similar to that of RIB: a BTB/POZ domain at the N-terminus and a coiled-coil at the C terminus (Sakai et al., 2000). One family member, RPT2, appears to respond to signals that promote phototropism. RPT2 also contains an NLS; however, it is not yet known where RPT2 functions. Based on the four putative NLSs, we speculate that RIB may function in the nucleus, where it would be positioned to regulate the expression of genes required for cytoskeletal changes during morphogenesis. Alternatively, RIB may reside in the cytoplasm and more directly regulate cytoskeletal organization. Since BTB/POZ domains can heterodimerize, RIB may have a partner(s) providing additional functional motifs. rib RNA is expressed in a dynamic pattern during development, including expression in cells that appear phenotypically normal in rib embryos. Thus, rib function is likely to be post-transcriptionally regulated, perhaps through the phosphorylation of its MAPK sites or through limited expression or activation of cofactors.

Further investigation of rib may help us to better understand the mechanisms by which cells control the direction of migration during development.

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