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# Ribbon modulates apical membrane during tube elongation through Crumbs and Moesin

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ABSTRACT

Although the formation and maintenance of epithelial tubes are essential for the viability of multicellular organisms, our understanding of the molecular and cellular events coordinating tubulogenesis is relatively limited. Here, we focus on the activities of Ribbon, a novel BTB-domain containing nuclear protein, in the elongation of two epithelial tubes: the *Drosophila* salivary gland and trachea. We show that Ribbon interacts with Lola Like, another BTB-domain containing protein required for robust nuclear localization of Ribbon, to upregulate *crumbs* expression and downregulate Moesin activity. Our ultrastructural analysis of *ribbon* null salivary glands by TEM reveals a diminished pool of subapical vesicles and an increase in microvillar structure, cellular changes consistent with the known role of Crumbs in apical membrane generation and of Moesin in the cross-linking of the apical membrane to the subapical cytoskeleton. Furthermore, the subapical localization of Rab11, a small GTPase associated with apical membrane delivery and rearrangement, is significantly diminished in *ribbon* mutant salivary glands and tracheae. These findings suggest that Ribbon and Lola Like function as a novel transcriptional cassette coordinating molecular changes at the apical membrane of epithelial cells to facilitate tube elongation.

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## Introduction

Tubular organs are essential for life in all multicellular organisms, including humans. Examples of tubular organs include the lungs and vasculature, which provide for gas and nutrient exchange, the kidneys, which maintain homeostatic fluid balance, the exocrine pancreas, which secretes enzymes for digestion, and the endocrine pancreas, which secretes hormones that regulate blood glucose levels and growth. Organs such as the salivary glands and lacrimal glands, which provide lubrication and secrete anti-microbial peptides, are also organized into tubular epithelial structures. Not surprisingly, defects in tube formation and/or maintenance from birth defects such as spina bifida to vascular diseases such as atherosclerosis and arterial thrombosis account for a huge proportion of the health care problems in developed nations. Despite the importance of tube formation and maintenance in human development and disease, our understanding of how tubular organ size and shape are established is quite limited, although recent studies in model organisms suggest key roles for regulated apical secretion and modifications of apical secretions in both diametrical tube expansion and tube length control (Seshaiah et al., 2001; Tsarouhas et al., 2007).

The *Drosophila* salivary gland (SG), which comprises two simple unbranched tubes specialized for secretion, and trachea (TR), a highly branched tubular network specialized for oxygen delivery, have become useful models for elucidating the molecular and cellular mechanisms of epithelial tube morphogenesis (Kerman et al., 2006). The SG forms from two placodes of polarized epithelial cells found in an anterior region of the embryo. Through regulated, sequential cell shape changes, the cells internalize to form paired tubular organs that elongate and migrate along several tissues to attain their final correct position in the embryo (Bradley et al., 2003; Myat and Andrew, 2000a, b; Vining et al., 2005). The trachea arises from 20 smaller placodes, ten on each side, of polarized epithelial cells. The tracheal primordia invaginate through a combination of cell shape changes and rearrangement to form internalized tracheal sacs (Nishimura et al., 2007). Subsets of cells within each sac then migrate in stereotypical directions to form branched structures that ultimately fuse to form a contiguous tubular network that carries oxygen from two posterior openings to all of the cells in the animal (Manning and Krasnow, 1993). Several transcription factors and their transcriptional targets have been implicated in either SG or TR morphogenesis. Fork head and Huckebein are transcription factors required for early stages of SG invagination and tube elongation, respectively, whereas the transcription factor Trachealess and its downstream target Breathless, an FGF receptor, are critical for tracheal invagination and subsequent branch migration, respectively (Isaac and Andrew, 1996; Klämbt et al., 1992;

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Myat and Andrew, 2000a,b; Weigel et al., 1989; Wilk et al., 1996; Zelzer and Shilo, 2000).

*ribbon* (*rib*) is required for the morphogenesis of many embryonic tissues including both the SG and TR (Bradley and Andrew, 2001; Jack and Myette, 1997; Shim et al., 2001). In *rib* mutants, the SG forms a tube that fails to elongate, and the TR exhibits delayed migration, ultimately failing to form a subset of branches, most notably the dorsal trunk (DT), which is the major artery connecting the trachea along the anterior–posterior body axis (Bradley and Andrew, 2001; Shim et al., 2001). *rib* encodes a 661-residue protein of the poorly understood Broad Tramtrack Bric-a-brac (BTB) domain transcription factor family that is composed of a BTB-domain, a Pipsqueak DNA-binding domain, nuclear localization sequences, putative MAPK phosphorylation sites, and a coiled-coil motif (Bradley and Andrew, 2001; Shim et al., 2001). The molecular basis for the *rib* mutant phenotype is unknown, but rescue experiments have shown that Rib functions in a tissue-autonomous manner (Bradley and Andrew, 2001).

The identification of interacting molecules as well as downstream target genes is key to understanding how Rib mediates tube elongation. Indeed, at least two transcriptional targets of Rib have been identified through genome-wide screens: *serpentine* (*serp*) and *vermiform* (*verm*) (Luschnig et al., 2006). These neighboring genes, whose full level of expression in late embryonic stages requires *rib*, encode proteins that are secreted into the lumen and contain chitin binding and deacetylation domains. Interestingly, the loss of these genes does not give rise to Rib-like TR defects; instead their loss results in excessively long tracheal tubes (Luschnig et al., 2006; Wang et al., 2006), a defect opposite to that observed in the TR and SG of *rib* mutants. Thus, the identification of *serp* and *verm* as Rib target genes is likely revealing later roles for Rib in limiting tube size. Here, we focus on the earlier role of Rib in tube elongation in the SG and TR dorsal trunk. We identify and characterize the role of a Rib interacting protein known as Lola Like (Lolal) in the SG and TR and we identify key downstream molecular targets that mediate tube elongation.

## Materials and methods

### *Fly strains and genetics*

Flies were grown and kept on standard cornmeal/agar medium. The fly lines used are given in Table 1. The UAS-Gal4 expression system (Brand and Perrimon, 1993) was used for tissue-specific expression. *p*-values were calculated using an online chi-square test calculator (Preacher, 2001).

### *Immunohistochemistry and in situ hybridization*

Embryo fixation and staining were performed as described previously (Reuter et al., 1990). Antibodies used are given in Table 2. Whole-mount in situ hybridizations were performed as described previously (Lehmann and Tautz, 1994). Embryos were visualized by Nomarski optics using a Zeiss Axiophot microscope or by fluorescence using a Zeiss LSM510 microscope. For protein level comparisons, images were taken under identical optical and electronic conditions. Pixel intensities were analyzed using Zeiss image acquisition software and calculations were performed in Excel (Microsoft).

### *Real-time PCR*

Homozygous embryos were selected by lack of GFP expression through an automated embryo sorter (Union Biometrica), confirmed visually on a dissecting microscope, and frozen in liquid nitrogen. mRNA was isolated using a Micro-FastTrack 2.0 kit (Invitrogen). *crumbs* (*crb*) mRNA levels were analyzed quantitatively by real-time PCR performed in triplicate using SuperScript II reverse transcriptase (Invitrogen), iQ SYBR Green Supermix (Biorad), and the iQ5 real-time PCR detection system (Biorad), and calculations were performed using the Pfaffl Method (Pfaffl, 2001). Gene-specific primer sequences used for quantification of *crb* transcript were: 5′-GATCGCGCGGCAA-GCATATT-3′ (forward) and 5′-GGTCCATCCAGAAGGCAACC-3′ (reverse). Reference control was eIF-4a transcript, amplified using the primer pair: 5′-TCGGTAATCTTCTGCAA-CACCCGT-3′ (forward) and 5′-CATCAATACCGCGCGCCAGTAAAT-3′ (reverse).

### *Transmission electron microscopy*

Wild-type (WT) and homozygous *rib* embryos were selected, processed for TEM and analyzed on a Philips EM120 as previously described (Myat and Andrew, 2000a). Thin sections were acquired throughout the lumena of three WT and three *rib* stage 12 SGs, and sections were analyzed at magnifications from 980× to 15000×.

**Table 1**
The references/sources of fly lines used in this study

| Fly line | Reference/source |
|---|---|
| *Oregon R* | WT control |
| *rib*$^{1}$ | Bloomington Stock Center |
| *rib*$^{2}$ | Bloomington Stock Center |
| *lolal*$^{l(2)k02515}$ | Bloomington Stock Center |
| *rib*$^{P7}$ | Shim et al., 2001 |
| *Df(2R)rib*$^{ex12}$ | Shim et al., 2001 |
| *lolal*$^{l(2)k02515}$*; da-Gal4, UAS-lolalGFP*/TM3,Sb | Faucheux et al., 2003 |
| *UAS-crb*$^{FL}$ | Wodarz et al., 1995 |
| *UAS-crb*$^{IC}$ | Wodarz et al., 1995 |
| *UAS-moe* $^{T559D}$*-myc* | Karagiosis and Ready, 2004 |
| *UAS-moe* $^{T559A}$ | Speck et al., 2003 |
| *btl-Gal4* | Shiga et al., 1996 |
| *fkh-Gal4* | Henderson and Andrew, 2000 |
| *UAS-moe-myc* | Karagiosis and Ready, 2004 |

**Table 2**
Antibodies and antibody dilutions used in this study

| Antibody | Produced in | Dilution[a] | Reference/Source |
|---|---|---|---|
| Actin | Mouse | 1:2000 | MP Biomedicals, Inc. |
| Avl | Chicken | 1:500 | Lu and Bilder 2005 |
| Crb | Mouse | 1:10 | DSHB[b] |
| Csp | Mouse | 1:40 | DSHB[c] |
| GFP | Mouse | 1:200 | Molecular Probes |
| GFP | Rabbit | 1:40,000/1:10,000 | Molecular Probes |
| pMoe | Rabbit | 1:500 | Karagiosis and Ready, 2004 |
| Rab11 | Rabbit | 1:500 | Satoh et al., 2005 |
| Rib | Rat | 1:50 | P. Bradley and D. Andrew, unpublished |
| SAS | Rabbit | 1:5000/1:500 | E. Organ and D. Cavener, unpublished |
| β-gal | Mouse | 1:10,000/1:500 | Promega |
| α-Spec Moe | Mouse Rabbit | 1:2 1:5000 | DSHB[d] Edwards et al., 1997 |

[a] When appropriate, dilutions used both for HRP and fluorescence stainings are provided, respectively. Otherwise, only the dilution used for fluorescence staining is provided.
[b] The monoclonal antibody CQ4 developed by Elisabeth Knust was obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.
[c] The monoclonal antibody 6D6 developed by Seymour Benzer was also obtained from DSHB.
Appropriate secondary antibodies conjugated to biotin (Vector Labs), Alexa Fluor 488, 555, 568, 647, or Rhodamine (Molecular Probes) were used in 1:500 dilution.
[d] The monoclonal antibody 3A9 developed by Daniel Branton and Ron Dubreuil was also obtained from DSHB.

## Results

### *Lola serves as a novel partner for Rib in SG and TR morphogenesis*

To choose the best *rib* allelic combination and to resolve discrepancies regarding the reported *rib* mutant defects, we evaluated the severity of SG and TR defects in the alleles *rib*$^{P7}$ and *rib*$^{1}$, which encode premature stops at residues 22 and 283, respectively, and in the deficiency *Df(2R)rib*$^{ex12}$, which removes *rib* and at least 25 other genes (Bradley and Andrew, 2001; Shim et al., 2001). Whereas wild-type (WT) stage 12 SGs turned and migrated at a high frequency, SGs of all *rib* alleles frequently failed to turn and migrate (Figs. 1A–E). In addition, whereas the WT stage 14 TR dorsal trunk (DT) extended and fused at a high frequency, DTs of all *rib* alleles exhibited only partial extension at this stage (Figs. 1H–L). Both *rib*$^{P7}$ and *rib*$^{1}$ TR extended relatively normal dorsal (Figs. 1H–L) and visceral (data not shown) branches as reported by Bradley and Andrew (2001), indicating that *rib* is not absolutely required for primary branch formation as

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