Ribon regulates morphogenesis of the *Drosophila* embryonic salivary gland through transcriptional activation and repression

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A B S T R A C T

Transcription factors affect spatiotemporal patterns of gene expression often regulating multiple aspects of tissue morphogenesis, including cell-type specification, cell proliferation, cell death, cell polarity, cell shape, cell arrangement and cell migration. In this work, we describe a distinct role for Ribon (Rib) in controlling cell shape/volume increases during elongation of the Drosophila salivary gland (SG). Notably, the morphogenetic changes in rib mutants occurred without effects on general SG cell attributes such as specification, proliferation and apoptosis. Moreover, the changes in cell shape/volume in rib mutants occurred without compromising epithelial-specific morphological attributes such as apicobasal polarity and junctional integrity. To identify the genes regulated by Rib, we performed ChIP-seq analysis in embryos driving expression of GFP-tagged Rib specifically in the SGs. To learn if the Rib binding sites identified in the ChIP-seq analysis were linked to changes in gene expression, we performed microarray analysis comparing RNA samples from age-matched wild-type and rib null embryos. From the superposed ChIP-seq and microarray gene expression data, we identified 60 genomic sites bound by Rib likely to regulate SG-specific gene expression. We confirmed several of the identified Rib targets by qRT-PCR and/or in situ hybridization. Our results indicate that Rib regulates cell growth and tissue shape in the *Drosophila* salivary gland via a diverse array of targets through both transcriptional activation and repression. Furthermore, our results suggest that autoregulation of rib expression may be a key component of the SG morphogenetic gene network.

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1. Introduction

Construction of a functionally specialized epithelial organ engages multiple aspects of cell structure and function. During the early stages of epithelial organ development, the regulation of general cell attributes such as specification and proliferation are critical (Inman et al., 2015; Maeda et al., 2007; Pan and Wright, 2011; Rawlins, 2011). Following specification, regulation of epithelial cell-type specific attributes such as integrity and polarity are required for tissue organization (Bryant and Mostov, 2008; Chung and Andrew, 2008; Laprise et al., 2009; Sotillos et al., 2013; Wang et al., 2012). Regulation of morphogenetic attributes such as changes in cell shape, orientation and migration are essential to define overall morphology and to achieve correct organ positioning (Andrew and Ewald, 2010).

Transcriptional regulation is the premier mechanism used by cells to control the differential gene expression that drives key steps during organogenesis. To understand the regulatory architecture at work during various stages of epithelial organogenesis, it is necessary to identify the targets of transcription factors that orchestrate organ development. Since transcription factors often impact multiple aspects of cell behavior that contribute to organogenesis, it is rare, if not impossible, to ascribe a selective role for any factor. For example, the bHLH transcription factor Ptf1a functions in multiple steps in specification and differentiation of pancreatic acinar cells (Cleveland et al., 2012). It is expressed and required in multipotent pancreatic progenitor cells, ultimately becoming restricted to the tip cells, where it is required for differentiation of the acini of the exocrine pancreas. In the differentiated acinar cells, Ptf1a directly binds and activates expression of the enzyme genes specific to the exocrine pancreas. Similarly, myogenic regulatory factors (Mrfs, including Myf5, MyoD, Mrf4) function early in myogenic precursor cells as muscle cell fate determinants in a partially redundant manner (Comai and Tajbakhsh, 2014). Different combinations of these same Mrfs are later also required for muscle differentiation where they directly regulate
expression of genes directing myofiber formation and the structural proteins required for muscle function.

Development of the embryonic salivary gland in *Drosophila* melanogaster has been well characterized (Chung et al., 2014). After specification of the salivary gland primordia, the cells of the placode invaginate and form an incipient tube (Myat and Andrew, 2000a, b). An elaborate set of cell shape changes and rearrangements enable tube elongation, which occurs simultaneously as migration of the epithelial collective positions the organ along the anteroposterior axis by stage 15 (Chung and Andrew, 2014; Kerman et al., 2008; Myat and Andrew, 2002). Following cell specification, both of these remains constant; tissue growth occurs through increases in cell size rather than cell number. In addition, cell polarity is maintained throughout the duration of tube formation, elongation and migration. For these reasons, the Drosophila salivary gland is an attractive system to study organogenesis and, in particular, to track morphogenesis (Chung et al., 2014; Girdler and Roper, 2014).

Several transcription factors have been characterized for their roles in organogenesis and functional specialization of the embryonic Drosophila salivary gland (Abrams and Andrew, 2005; Abrams et al., 2006; Bradley and Andrew, 2001; Fox et al., 2010; Fox et al., 2013; Myat and Andrew, 2000a, b, 2002). Among these factors is Ribon (Rib), a broadly expressed BTB domain protein, required for both salivary gland and tracheal tube elongation (Bradley and Andrew, 2001; Shim et al., 2001). The tube elongation defects in *rib* mutants have been linked to delays in apical/lumenal expansion, with the salivary gland lumen ultimately achieving only 60% the length of the WT lumen (Cheshire et al., 2008). Consistent with increases in apical domain stiffness, loss of *rib* is linked to reduced expression of the gene encoding the apical domain determinant Crumbs (Crb), increased levels of active, phosphorylated Moesin (Moe), an apically-localized ERM family protein linked to reduced expression of the gene encoding the apical determinant Crumbs (Crb), increased levels of active, phosphorylated Moesin (Moe), an apically-localized ERM family protein linked to reduced expression of the gene encoding the apical do-

### 2. Materials and methods

#### 2.1. Fly strains

Oregon R embryos were used as the wild-type control in all experiments. The trans-allelic combination of *rib* 
(Bradley et al., 2001) and *rib* 
(Shim et al., 2001) was used to analyze *rib* mutant phenotypes and gene expression changes by microarray, qRT-PCR and whole mount in situ hybridization. The *rib* alleles are EMS-induced mutations induced at different times and in different labs; *rib* has a premature stop codon introduced at residue 22 and *rib* has a stop codon introduced at residue 283. UAS-*rib-GFP* was built by cloning a PCR amplification of the full-length *rib* ORF into the pENTR-D vector and subsequent gateway cloning into the pTWG vector, placing the entire GFP coding region downstream of and in frame with the Rib ORF. The following lines were generated and crossed together to test for rescue of *rib* mutant SG phenotypes: *ribP7* [Klatt et al., 2004] and *sage-Gal4* expression driven by *fkh-Gal4* and *sage-Gal4* was used to determine the full set of cell types where these drivers are active.

#### 2.2. Immunohistochemistry and in situ hybridization

Embryo fixation and immunostaining were performed as described (Reuter and Scott, 1990). The primary antibodies used included rabbit α-SAS (D. Cavener, 1:500), guinea pig α-Sage (1:100), rabbit α-Forkhead (S. Beckendorf, 1:500), rat α-Creba (1:1000), rat α-Pasilla (1:5000), rabbit α-Phospho histone H3 (Abcam, 1:500), rabbit α-CC (Cell Signaling, 1:100), α-PKζ (C-20, Santa Cruz Biotech, 1:500), rat α-DE-Cadherin (DCAD2, DSHB, 1:100), mouse α-Coracle (C6566.9, DSHB, 1:200), mouse α-alpha-spectrin (3A9, DSHB, 1:1), rabbit α-Laminin (J. Fessler, 1:1000), mouse α-DCSP-2 (6D6, DSHB, 1:200), mouse α-Lamin (ADL84.12, DSHB, 1:200), mouse α-beta-gal (Promega, 1:1000) and rabbit α-gal (Molecular probes, 1:1000). For HRP staining, Biotin-labeled secondary antibodies were used at 1:500 dilution (Molecular Probes). The HRP staining signal was amplified using the Vectastain ABC kit (Vector Labs). For fluorescence staining, Alexa-488- or Alexa-568- or Alexa-647-labeled secondary antibodies were used at 1:500 dilution (Molecular Probes). Confocal images were obtained using the LSM 700 confocal microscope (Carl Zeiss, Inc.). Whole-mount in situ hybridization was performed as described previously (Lehmann and Tautz, 1994). Images from in situ hybridized and HRP stained embryos were obtained with an Axiophot microscope (Carl Zeiss, Inc.).

#### 2.3. Chromatin immunoprecipitation and deep sequencing

Chromatin extraction and immunoprecipitation were performed as described previously (Negre et al., 2006). Briefly, chromatin from three independent collections of stage 11–16 *fkh-Gal4::UAS-rib-GFP* embryos and three of *sage-Gal4::UAS-rib-GFP* embryos were cross-linked at room temperature in 1.8% formaldehyde in 2 ml of homogenization buffer (50 mM KCl, 15 mM NaCl, 15 mM HEPES [pH 7.6], 4 mM MgCl₂, 0.5 mM DTT, 0.5% Triton X-100 and cComplete protease inhibitor cocktail [1 tablet per 50 ml buffer]). The cross-linked material was resuspended in 0.1% SDS and 0.5% N-lauroylsarcosine in 0.5 ml lysis buffer (140 mM NaCl, 15 mM HEPES [pH 7.6], 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 1% Triton X-100, 0.5 mM DTT and cComplete protease inhibitor cocktail [1 tablet per 50 ml buffer]). Chromatin was sonicated three times at 4°C using the Sonic Dismembrator Model 100 (Fisher Scientific) under the following conditions: power setting 3, 20 s ON, 20 s OFF. Immediately after sonication, the chromatin extract was stored at −80°C prior to immunoprecipitation. Immunoprecipitations were performed as described in (Negre et al., 2006), using a polyclonal goat anti-GFP antibody (a gift from Kevin White). Immunoprecipitated DNA was prepared for Illumina sequencing using the TrueSeq ChIP Sample Prep Kit (Illumina) and sequenced on an Illumina HiSeq 2000.
using the Spot (Irizarry et al., 2003a, b) and statistical analyses were performed using standard Affymetrix protocols. Three samples for each genotype were hybridized to Drosophila Genome 2.0 Chips. Scanning was carried out using confocal image sections using Adobe Photoshop Elements 6.0. Cross-sectional nuclei were counted based on the following criteria: (1) presence of DAPI+ DNA, (2) connectivity of cells to the lumen and (3) strong Lamin signal separating individual DAPI+ nuclei. Manual surface renderings of the SG in the YZ-plane and the associated volumetric analyses were performed using Imaris x64 version 7.7.2 (Bitplane, Oxford Instruments).

2.9. Motif analysis

The SeqPos tool from the Cistrome Galaxy-based platform (http://cistrome.org/ap/) was used to identify enriched motifs in ChIP-seq peaks (He et al., 2010; Liu et al., 2011).

2.10. DAVID analysis

Functional clustering of Rib targets from microarray and ChIP-seq data were performed to place them under gene ontological categories (GO terms) according to the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (Huang da et al., 2009a, b).

3. Results

3.1. Salivary gland cells are properly specified in rib mutant embryos

Embryos missing rib function (rib1/ribP7) have severe defects in overall SG morphology (Fig. 1A’ and B’). SGs are shorter, have irregular shapes as well as bulging lumens at late embryonic stages. We asked if these abnormalities could be due to changes in specification, proliferation or apoptosis of SG cells. Immunofluorescence staining of the SG with several SG-expressed nuclear function (rib1/ribP7) have severe defects in overall SG morphology (Fig. 1A’ and B’). SGs are shorter, have irregular shapes as well as bulging lumens at late embryonic stages. We asked if these abnormalities could be due to changes in specification, proliferation or apoptosis of SG cells. Immunofluorescence staining of the SG with several SG-expressed nuclear function (rib1/ribP7) have severe defects in overall SG morphology (Fig. 1A’ and B’). SGs are shorter, have irregular shapes as well as bulging lumens at late embryonic stages. We asked if these abnormalities could be due to changes in specification, proliferation or apoptosis of SG cells. Immunofluorescence staining of the SG with several SG-expressed nuclear proteins, including the bHLH transcription factor Sage (Fig. 1C and G), the winged-helix FoxA transcription factor Fork head (Fkh) (Fig. 1D and H), the bZIP transcription factor CrebA (Fig. 1E and I), and the nuclear RNA binding protein Pasilla (Ps) (Fig. 1F and J) revealed similar staining in wild-type and rib mutant SGs. Once specified, WT SG cells do not undergo additional cell divisions: the tissue grows by increase in cell size, not cell number (Myat and Andrew, 2000a; Sonnenblick, 1950). Staining with phospho-histone H3, a marker for dividing cells, indicated that there were no cryptic cell divisions in the rib mutant glands (Fig. 1K and L). Also, apoptosis does not occur during normal SG development (Ismat et al., 2013; Myat and Andrew, 2000a). Cleaved caspase 3 staining (Fig. 1M–N”) and N–N*) showed no evidence for cell death in rib mutant or WT SGs. Furthermore, cell counts revealed no significant difference in the number of cells in the rib1/ribP7 SGs compared with WT SGs (Fig. 1O). Collectively, these results indicate that the defective SG morphology in rib mutants is not caused by abnormalities in general cell attributes such as cell size and/or SG expression according to the BDGP release 6 (dm6, August 2014) using Burrows-Wheeler Alignment (BWA) tool with default parameters (dos Santos et al., 2015; Li and Durbin, 2009; St Pierre et al., 2014). Sequencing reads from biological replicates were combined after mapping using Picard (http://broadinstitute.github.io/picard) and the MACS (v2) peak caller was used to identify and score peaks. (Zhang et al., 2008). Peak calling was carried out using the following MACS parameters (P-value: 10e-5; mfold: 10, 32), comparing ChIP DNA to matching input control samples.

2.5. Microarray gene expression analysis

Three independent collections of stage 11–16 rib1/ribP7 embryos and three of wild-type embryos were isolated using a COPAS Select embryo sorter (Union Biometrica). Total RNA was isolated by Trizol (Invitrogen) extraction and cleaned up with the Qiagen RNeasy kit (Qiagen). Total RNA (100 ng) was labeled and amplified using standard Affymetrix protocols. Three samples for each genotype were hybridized to Drosophila Genome 2.0 Chips. Scanned intensity values were normalized using RNA (Partek software (Irizarry et al., 2003a, b) and statistical analyses were performed using the Spotfire software package (TIBCO). Target genes were identified as those that were upregulated/downregulated (± 1.5-fold change cutoff, P ≤ 0.05) in rib1/ribP7 embryos when compared with Oregon R controls. Microarray data are also available from GEO (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE73781.

2.6. Real-time quantitative PCR (RT-qPCR)

Total RNA from three independent samples each of wild-type and rib1/ribP7 mutant embryos (stage 11–16) was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) for two-step qPCR reactions with oligo(dT)18 primers (IDT). Gene-specific primers were used to amplify the corresponding CDNA by RT-qPCR on a CFX96 Real-Time PCR Detection System (BioRad) using SYBR Green Supermix (BioRad). Primer sets for each putative gene were chosen for RT-qPCR based on their fold change cutoff, 1.5-fold change and statistically significant (P < 0.0021) as well as the number of overlaps in Rib binding sites and genes downregulated (P = 0.5537) or upregulated (P = 0.0486) in rib mutants based on microarray analysis. A total estimate of 18,000 Drosophila encoded genes was used for this analysis.

2.8. Image processing

Nuclear counts were performed on confocal image sections using Adobe Photoshop Elements 6.0. Cross-sectional nuclei were counted based on the following criteria: (1) presence of DAPI+ DNA, (2) connectivity of cells to the lumen and (3) strong Lamin signal separating individual DAPI+ nuclei. Manual surface renderings of the SG in the YZ-plane and the associated volumetric analyses were performed using Imaris x64 version 7.7.2 (Bitplane, Oxford Instruments).

2.9. Motif analysis

The SeqPos tool from the Cistrome Galaxy-based platform (http://cistrome.org/ap/) was used to identify enriched motifs in ChIP-seq peaks (He et al., 2010; Liu et al., 2011).

2.10. DAVID analysis

Functional clustering of Rib targets from microarray and ChIP-seq data were performed to place them under gene ontological categories (GO terms) according to the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (Huang da et al., 2009a, b).

2.11. Statistical analysis

Statistical analyses were carried out using SigmaPlot 11.0 (Sysstat software Inc.).
Fig. 1. Cell specification, cell division and apoptosis are not affected in rib mutant salivary glands (SGs). (A, B) Wild-type (WT) and rib1/ribP7 embryos stained for the apical cell membrane protein SAS and DAPI show overall embryo morphology. (A') A high magnification image of the WT SG (white box in A) reveals an elongated SG tube. (B') A high magnification image of the rib1/ribP7 SG (white box in B) reveals a short, wide SG tube. (C–J) WT and rib mutant SGs were stained with DAPI and four different cell-specification markers: Sage (C, G), Fkh (D, H), CrebA (E, I), and Ps (F, J). (K, L) WT and rib mutant salivary glands were stained with DAPI and phospho-histone H3 (pH3) antiserum to assay for cell division. The white arrowheads indicate one of the many pH3+ neuroblasts undergoing cell division. (K'–K''' and L'–L''' high-magnification images of WT and rib mutant SGs (white boxes in K and L, respectively) are shown. (M, N) WT and rib mutant SGs were stained with DAPI and cleaved caspase3 antibody to assay for apoptosis. The yellow arrowheads indicate one of the many CC3+ intersegmental surface epithelial cells undergoing apoptosis. (M'–M''' and N'–N''' high-magnification images of WT and rib mutant SGs (white boxes in M and N, respectively) are shown. (O) Cell count from WT and rib mutants showed no statistically significant difference in the number of cells present in WT versus rib mutant SGs (n=10 glands). Scale bars: 20 μm.
Fig. 2. Cell junction integrity, cell polarity and secretory vesicle marker localization are not affected in rib mutant SGs. (A, B) WT and rib1/ribP7 SGs stained with aPKC (apical domain marker) and E-Cad (adherens junction (AJ) marker). Arrowheads indicate co-localization of markers and apical localization of AJs. (C, D) WT and rib1/ribP7 SGs stained with DAPI and Cora (septate junction (SJ) marker). Arrowheads indicate sub-apicolateral localization of SJs. (E, F) WT and rib1/ribP7 SGs stained with alpha-Spectrin (lateral membrane marker) and Sas (apical membrane marker). White and cyan arrowheads indicate distinct localization of the lateral and apical cell membrane domains, respectively. (G, H) WT and rib1/ribP7 SGs stained with DAPI and the basement membrane component laminin as an indirect marker for the basal cell domain. Arrowheads indicate basal deposition/localization of laminin. (I, J) WT and rib1/ribP7 SG stained with CSP2 (exocytic/secretory vesicle marker). Arrowheads indicate the apical enrichment of CSP2, implying functional secretion in rib mutant SG cells. Scale bars: 20 μm.
specification, cell division or cell death.

3.2. Salivary gland cell integrity, polarity and secretory potential are unaffected in rib mutants

Tissue integrity and polarity are critical determinants of epithelial organogenesis (Andrew and Ewald, 2010). As with other epithelia, tissue integrity is maintained in the developing SG epithelium by the adherens junction (AJ) and septate junction (SJ) complexes. Staining for E-Cad and other components of the AJ complex revealed their proper localization in rib mutant SGs (Fig. 2A and B; data not shown). Likewise, staining for SJ proteins, including Cora and Dig, revealed no differences between rib mutant and WT SGs (Fig. 2C and D; data not shown). SG cells maintain apicobasal polarity throughout organogenesis with the apical surface facing the developing gland lumen (Chung and Andrew, 2014). Staining for the apical polarity marker Sas and lateral polarity marker alpha-spectrin showed that the apicolateral polarity of SG cells in rib mutants is not compromised (Fig. 2E and F). This staining also confirmed the altered cell shape in rib mutants (cuboidal versus columnar in WT) that had been reported earlier (Blake et al., 1998). Moreover, basal cell polarity is also intact based on the presence of laminin deposits observed on the basal surfaces of both WT and rib mutant SGs (Fig. 2G and H). In glandular epithelia such as the SG, cell secretion and the resulting hydrostatic pressure can affect the normal trajectory of

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**Fig. 3.** Loss of rib affects cell volume but not cell arrangement. (A) Whole surface rendering of a WT stage 12 SG. (A′) Luminal surface rendering of the gland shown in A. (B) Whole surface rendering of a rib/ribP7 stage 12 SG. (B′) Luminal surface rendering of the gland shown in B. (C) The SG volume (difference between the whole volume and luminal volume) of the rib/ribP7 mutants was significantly decreased (t-test, P < 0.001) compared to the WT. (C′) The estimated mean cell volume (SG volume/total cell count) of the rib/ribP7 mutants was significantly decreased (t-test, P < 0.001) compared to the cell volume in the WT SG. (D) Representative scheme for obtaining cross-sections of the WT SG from three different regions along the length of the organ. Yellow dashed lines show Pr: proximal, Mi: middle and Di: distal sections. (D′–D‴) Cross-sections of the WT tube shown in (D) from the proximal, middle and distal regions with nuclear count labels. (E) Representative scheme for obtaining cross-sections of the rib/ribP7 SG from three different regions along the length of the organ. (E′–E‴) Cross-sections of the rib/ribP7 tube shown in (E) from the proximal, middle and distal regions with nuclear count labels. (F) The number of cells (mean ± SE) encircling the lumen was not significantly different (one-way ANOVA) between the WT and the rib/ribP7. Scale bars: 10 μm.
Fig. 4. ChIP-seq analysis identifies Rib binding sites in salivary gland cells. (A) Schematic outline of the experimental approach to identify SG-specific Rib binding sites. ChIP-seq datasets were obtained from samples using two different GAL4 constructs to drive expression of UAS-rib-gfp in the SG. The overlap of binding events observed with both drivers enriches for SG-specific Rib binding. (B) Rescue of the SG phenotype in the rib<sup>1</sup>/rib<sup>P7</sup> mutant background with fkh-Gal4::UAS-rib-GFP verified the functionality of UAS-rib-gfp construct used in the ChIP-seq experiments. (C) Tissue expression of fkh-GAL4 and sage-GAL4 drivers spanning the stages used for the ChIP-seq analyses. Arrowheads indicate the SG at different developmental stages. (D) SG-enriched ChIP-seq signals correspond to Rib binding events in the vicinity of two Rib target genes – Hsp70Ba and Obp99b.
Fig. 5. Microarray gene expression analysis indicates the direction of transcriptional control of Rib targets. (A) RNA was isolated from three individual samples each of stage 11–16 WT and rib1/ribP7 embryos. Volcano plot shows genes that were downregulated (blue) or upregulated (red) at least 1.5-fold \( (P < 0.05) \) in rib mutants compared to WT. In each direction, the top 20 annotated candidates, based on fold change, are marked (cyan). Transcripts with fold change values between \(-1.5\) and \(1.5\) \( (P > 0.05) \) are shown in yellow. Fold change values that are not statistically significant \( (P > 0.05) \) are indicated by gray. (B, C) Venn diagrams representing the overlap of 494 genes from the ChIP-seq and microarray (774 targets activated and 1176 repressed by Rib, respectively) data sets are shown. (D) The set of transcripts that are downregulated (blue) or upregulated (red) at least 1.5-fold \( (P < 0.05) \) in rib mutants compared to WT and testing positive for SG Rib binding (ChIP-seq analyses) are marked (cyan). (E, F) qRT-PCR results for a subset of genes obtained from the overlap of ChIP-seq and microarray data confirms significant expression change in the same direction as observed with microarray analysis for all but two examples, Sema-5C and CLS. \( *P < 0.01, **P < 0.001 \), Mann–Whitney U test.
organogenesis and hence gland morphology (Navis and Bagnat, 2015). Staining for the secretory vesicle marker CSP2 showed apical enrichment suggesting uncompromised secretory potential in rib mutant SGs (Fig. 2I and J). Taken together, these results suggest that the defective tube morphology in rib mutant SGs is not attributable to abnormal epithelial cell type-specific characteristics such as cell integrity, polarity or secretory potential.

3.3. rib mutant SGs are significantly smaller than WT SGs

To determine if the defective morphology of rib mutant salivary glands is due to changes in cell size/shape and, consequently its volume, or is due to changes in the arrangement of cells around the lumen, we performed morphometric analyses. For volumetry, we obtained manual surface renderings of the whole SG tissue along the YZ-axis (Fig. 3A and B). The volumes obtained from luminal surface renderings (Fig. 3A' and B') were then subtracted from the whole SG volumes to obtain the total cellular volume of the gland, termed SG volume. The mean SG volume in the rib+ /rib+mutants was significantly decreased compared to the age-matched WT embryos (Fig. 3C). We also estimated the cell volume by dividing the individual SG volume by the total number of cells within each gland. The result of these estimates showed a significant decrease in the mean cell volume of rib+/rib+ mutant SGs; cells in the rib mutant glands attained only 53% of the WT cell volume (Fig. 3C). Despite the overt differences in cell and organ size, nuclear size, based on DAPI and Lamin staining, did not appear to be different between WT and rib mutant SGs (Fig. 3D and E). Since SG shape is also dependent on how the cells are arranged around the lumen (Chung and Andrew, 2014; Pirraglia et al., 2013; Xu et al., 2011), we analyzed cell arrangement by examining the cross-sectional images obtained along the luminal axis (Fig. 3D, E). We found no significant difference in cell arrangement between the rib+/rib+ mutant and WT SGs (Fig. 3D–E′, E–E′ and F). Overall, these results suggest that a major morphological defect in rib mutant SGs is the significant decrease in average cell size/volume. Our findings also rule out a role for abnormal cell arrangement in contributing to the defective SG morphology of rib mutants.

3.4. ChIP-seq analysis reveals direct binding targets of Rib in the salivary gland

To identify direct targets of Rib in the developing SG, we performed ChIP-seq analysis. We used the fkh-GAL4 and sage-GAL4 drivers to obtain parallel ChIP-seq data sets (Fig. 4A). The C-terminal GFP-tagged UAS-Rib construct, which was driven by the two GAL4 drivers, was functional, as it rescued the SG phenotype in the rib mutant background (Fig. 4B). We processed deep sequencing data from both driver conditions, and superimposed the datasets to enrich for SG-specific Rib binding sites. The fkh-GAL4 and sage-GAL4 constructs each drive strong expression of a UAS-lacZ reporter in the salivary gland as well as a variety of other tissues (Fig. 4C). Analysis of the individual expression patterns from these drivers indicates that they share high-level salivary gland expression. Hence, identification of binding events common to both data sets (fkh-GAL4::UAS-rib-GFP and sage-GAL4::UAS-rib-GFP), as shown for representative genes Hsp70Ba and Obp99b (Fig. 4D), should reveal Rib-binding sites in the SG. Using this strategy, we identified 494 genes bound and, hence, potentially directly regulated by Rib in the developing SG (Supplementary Table 1).

3.5. Rib functions as both a transcriptional activator and repressor (activation/repression) of potential targets by Rib, we performed microarray gene expression analysis in both WT and rib+/rib+ embryos spanning the stages of SG organogenesis (Fig. 5A). Microarray analysis revealed that 774 transcripts were down-regulated in the rib+/rib+mutants compared to WT embryos (fold change ≤ 1.5 ×, P ≤ 0.05), whereas 1176 transcripts were upregulated at least 1.5 × (P ≤ 0.05) in the rib+/rib+ mutants compared to WT embryos (Supplementary Tables 2 and 3). Intersection of the microarray gene expression results with the SG-specific ChIP-seq data sets resulted in 20 candidates for direct Rib activation and 40 candidates for direct Rib repression in the SG (Fig. 5B–D), a number consistent with chance for the activated genes (P = 0.5537) but that is higher than expected by chance for the repressed genes (P = 0.0486). A subset of these candidates was further validated by qRT-PCR analysis (Fig. 5E and F). Of the 15 candidates chosen for qRT-PCR verification, 13 showed significant change in expression levels consistent with the directionality of change suggested by the microarray gene expression analysis. Notable among the candidate targets identified by our analysis for Rib regulation is rib itself, suggesting the possibility of autoregulation. Unlike most genes represented on the Affymetrix array chips, the gene Prosap was covered by two sets of probes (one corresponding to the 5′ end near the known mapped promoter and one corresponding to the 3′ end near the coding exons), resulting in discordant results for regulation of this gene in the microarray analysis. Since the array is biased toward 3′ measures of gene expression and since Rib occupancy mapped just upstream of the 3′ probes, a 3′ region was used for the qRT-PCR analysis. The qRT-PCR verified the microarray findings for the 3′ region of the gene; Prosap expression was significantly increased in the rib+/rib+ embryos compared to controls, consistent with Rib repression.

Functional clustering of potential Rib targets by DAVID gene ontological (GO) classification was done on the following four data sets: (1) List of genes activated by Rib in the whole embryo, (2) List of genes repressed by Rib in the whole embryo, (3) List of genes activated by Rib in the whole embryo and bound by Rib in the SG, and (4) List of genes repressed by Rib in the whole embryo and bound by Rib in the SG. The results from functional annotation clusters with a significant (P ≤ 0.05) enrichment score of at least 2.0 (Tables 1–4) showed that the targets activated by Rib in the whole embryo were generally grouped under the GO terms: DNA-related biological processes (including DNA replication and repair), nuclear chromosome part, nuclear lumen and cellular stress response. The candidate direct SG-specific targets of Rib were generally grouped under stress response, but included sub-categories of the spliceosome, endocytosis and nucleotide binding. The target genes repressed by Rib in the whole embryo included neurobiological processes and neuronal morphogenesis as well as cofactor/coenzyme binding and biosynthesis. Curiously, the GO term salivary gland morphogenesis was the predominant category for candidate direct SG-specific targets repressed by Rib. Taken together, these results indicate that the SG-specific Rib gene regulatory network is involved in the activation of processes linked to cellular stress response and repression of genes linked to morphogenesis. The finding that Rib regulates its own expression by auto-regulation — specifically, repression — suggests that Rib auto-repression may be a critical component of the regulatory architecture promoting morphogenesis during SG development.

3.6. Validation/examination of Rib targets in the salivary gland

Since genes whose expression may change in only a subset of tissues in rib mutants could be missed by microarray studies, we also examined potential regulation of known SG-expressed genes. Potential Rib target genes in the salivary gland were identified in the overlap of known SG-expressed genes from the BDGP database
### Table 1
Results from DAVID clustering analysis of GO terms for genes activated by Ribbon based on microarray data.

<table>
<thead>
<tr>
<th>Annotation cluster (enrichment score)</th>
<th>GO term</th>
<th>Fold enrichment</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>1 (5.34)</td>
<td>DNA metabolic process</td>
<td>3.5</td>
<td>1.0E-09</td>
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<td>DNA repair</td>
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### Table 2
Results from DAVID clustering analysis of GO terms for genes repressed by Ribbon based on microarray data.

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(Supplementary Table 4) and the Rib binding sites identified by ChIP-seq: there were twenty-seven such genes (a number higher than expected by pure chance; P=0.0021). Three-way overlaps of the microarray gene expression dataset, ChIP-seq dataset and the BDGP-identified SG-expressed genes produced sema-5c as a target activated by Rib in the SG (Fig. 6A) and qtc and CG5953 as targets
repressed by Rib in the SG (Fig. 6B). We validated the regulation of these targets and twelve others, which either had Rib binding sites or showed changes in the microarray analysis, by in situ hybridization analysis (Fig. 6C and D). Of the 15 putative targets we chose to examine by in situ hybridization, ten showed reduced SG expression in rib mutants, four showed elevated SG expression in rib mutants, and one showed no detectable change in the rib mutant SGs compared with those of the rib heterozygotes (Table 5; Supplementary Fig. 1). Notably, two of the putative BDGP targets that were chosen post-hoc-net and jbug-based on a peak-like chip signal in proximity to the gene, but were not called as peaks by MACS, also showed regulation by Rib. Furthermore, our in situ analysis also confirmed that rib is subject to autoregulation, and pasilla (ps), one of the putative targets and a SG specification marker used in our study (Fig. 1F and J), was not subject to Rib regulation. Overall, the in situ hybridization analysis confirms Rib as both a transcriptional activator and repressor in the developing SG, a role also confirmed by microarray gene expression analysis of whole embryos and further corroborated by identification of Rib binding sites by ChIP-seq.

3.7. Determination of Rib consensus binding motifs

The ChIP-seq data revealed 494 genes directly bound by Rib in the salivary gland (Fig. 4). A SeqPos-based motif analysis of the Rib binding sites associated with these genes allowed us to identify DNA sequences that occurred most frequently, potentially revealing consensus sites recognized by Rib and/or its DNA binding partners. An analysis of the top ten sequence motifs identified in this way revealed shared sequence cores within several of the motifs (Fig. 7). For example, motifs 1 and 4 share a CTATCT/CORE sequence, which is also the reverse complement of the six most conserved residues of motif 10. Likewise, motifs 2 and 5 share a GCACGG core sequence, and motifs 3 and 9 share a GCCGAC core sequence. It was not possible to learn if the different motifs were associated with gene activation versus repression because of the relatively smaller number of genes for which the direction of gene regulation is known or could be inferred based on either in situ or microarray data. Nonetheless, these sequences will be prime targets for future in vivo DNA binding studies and in vivo enhancer analyses, and may help to identify Rib partners that may function in gene activation versus repression.

4. Discussion

In this study, we asked which aspects of organ morphogenesis are affected by the loss of the BTB-containing nuclear protein Rib; our studies ruled out cell specification, cell death, cell division, junctional integrity, and cell polarity, as well as defects in the cell rearrangements that normally accompany SG internalization and tube elongation. Indeed, we discovered that a major morphological defect in rib mutants is the failure of SG cells to achieve the same size and shape as WT SG cells. rib mutant SG cells are, on average, only 53% the volume of WT cells, even though nuclear size appears no different. To learn which genes are directly regulated by Rib in the SGs, we carried out ChIP-Seq analysis using a strategy to specifically enrich for SG binding sites. Combining the ChIP-Seq findings with gene expression studies, we have identified an interesting set of Rib targets that are likely to support changes in cell shape and in cell/tissue growth in the SG. We have also learned that Rib acts as both a transcriptional activator and repressor, and represses its own expression. Finally, we have identified sequence motifs through which Rib and/or its binding partners control SG gene expression.

Previous studies of rib function in both the SG and trachea led to the conclusion that the rib loss-of-function defects were primarily in epithelial migration (Bradley and Andrew, 2001; Shim et al., 2001). The SGs of rib mutants failed to turn and migrate along the visceral mesoderm, as WT SGs do (Bradley and Andrew, 2001). Similarly, tracheal branches failed to elongate, with some branches having more severe defects than others (Bradley and Andrew, 2001; Shim et al., 2001), a phenotype that was linked by Shim et al. (2001) to FGF signaling. Subsequent live imaging studies revealed compromised tube elongation attributable to reduced apical membrane length and increased apical stiffness in both the SGs and trachea of rib mutants (Cheshire et al., 2008). The delayed and limited apical elongation (SGs only ever achieved 60% of WT length) was molecularly linked to reduced expression of the apical determinant crb, increased apical levels of active, phosphorylated Moe, as well as decreased apical accumulation of Rab11, a component of the apical recycling endosome (Kerman et al., 2008). These previous findings pointed to a role for Rib in affecting cell shape changes essential for SG morphogenesis at the

---

Table 3

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Table 4

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apical surface. In the current study, which takes advantage of newly available image analysis programs to characterize gland morphometry, we discovered that loss of Rib leads not only to a reduction in apical tube dimensions, its loss also significantly affects the volume of the entire gland and its constituent cells. Thus, the SG tube may simply be too small to fully contact the tissues upon which it normally migrates. Alternatively, the reduced cell size may limit the ability of SG cells to generate sufficient collective forces to elongate and simultaneously position the tubular organ along the anteroposterior axis.

The nuclear localization of Rib in all tissues, including the SG, and the possibility of its cooperation with another BTB domain-containing factor Lolal (a transcription factor) in epithelial cells suggests that Rib regulates gene transcription (Kerman et al., ...
Thus, we sought to determine the targets of Rib in the SG by ChIP-seq and we identified 494 genes with Rib binding sites. A first pass inspection of the genes associated with Rib binding in the salivary gland revealed twenty-three functional clusters based on DAVID (Supplementary Table 5). Notable GO terms identifying these gene clusters included ribosome, microtubule-based processes, cell adhesion, cell motion, cell projection morphogenesis, imaginal disc morphogenesis, cell fate commitment, epithelial morphogenesis, plasma membrane, salivary gland, tracheal, eye and muscle development, stem-cell maintenance and epithelial-
Fig. 7. The top ten consensus sites found within Rib bound DNA fragments from the ChIP-seq analysis reveal conserved core motifs. (A) Motif 1 and Motif 4 are displaced by a single nucleotide and Motif 10 is the reverse complement of the most conserved sequence within Motifs 1 and 4. (B and C) Similarly, Motifs 2 and 5 contain a shared core sequence, as do Motifs 3 and 9. (D–F) Motifs 6–8 are distinct, although the N-terminal portion of Motif 6 is related to Motifs 3 and 9. (G) Table of top 10 Motif clusters with the number of hits, the Cutoff, Z score, -log10(P value) and mean position is shown. (H) A hypothetical model of the Rib gene regulatory network in the context of SG development.
structure maintenance. These findings suggest that Rib may directly regulate cell size through translational regulation by affecting expression of genes encoding ribosomal proteins (Annotation cluster 1). The clustering analysis suggests that Rib probably also directly affects morphogenesis through effects on the cytoskeleton, cell adhesion and other morphogenetic events (Annotations clusters 2, 3, 4–6, 9–12). The David analysis also suggests that Rib could bind the same DNA sites in all tissues in which it is expressed and required, based on the finding that genes linked to the salivary gland (Annotation clusters 13, 15), trachea (Annotation cluster 19), eye (Annotation cluster 8), wing disc (Annotation clusters 4, 18), germ cells (Annotation cluster 14) and muscle cells (Annotation cluster 22) were found to be enriched in the sites bound by Rib in the SG. This final conclusion can be directly tested using a strategy similar to the one we used for the SG by doing the ChIP-seq analysis with Gal4 drivers expressed in one or more of these other tissues.

Combining the microarray gene expression analyses with the ChIP-seq results revealed that Rib functions in both transcriptional activation and repression, and provided evidence for Rib auto-regulation. The intersection of genes activated by Rib and Rib binding sites determined from ChIP-seq identified twenty putative targets activated by Rib in the SG. Similarly the overlap of genes repressed by Rib and ChIP-seq sites identified forty putative targets repressed by Rib in the SG. qRT-PCR on a subset of these putative targets confirmed the directionality of regulation of approximately 85% of the genes, further validating this unbiased approach to identifying Rib targets. The genes identified as targets for activation by Rib in the whole embryo spanned several GO categories such as DNA-related biological processes (including DNA replication and repair), nuclear chromosome part, nuclear lumen and cellular stress response, whereas the targets for repression by Rib in the whole embryo were categorized under neurobiological processes and neuronal morphogenesis as well as cofactor/coenzyme binding and biosynthesis. Meanwhile, the targets activated by Rib in the SC, identified by the two-way overlap, fell primarily under cellular stress response genes – most notably because this group included several heat shock protein (HSP) genes that require Rib for their high-level expression. This finding is again consistent with a direct role for Rib in cell volume increases. Although HSPs were initially identified because their expression increases under conditions of high temperature and other stresses, HSPs are now known to primarily function as chaperones to facilitate protein folding and block aggregation, a potential problem associated with high levels of protein production. Alternatively, cellular stress response genes have also been shown to promote cell migration and morphogenesis (Cobreros et al., 2008; Li et al., 2012). The potential direct targets repressed by Rib in the SG, came primarily under the GO term salivary gland morphogenesis. Altogether, this finding could suggest that Rib represses morphogenesis through its targets to allow for sufficient cell size increase (growth) prior to releasing the “brake” on morphogenesis through self-repression.

We also performed a three-way superposition of gene lists obtained from (1) ChIP-seq data, (2) Microarray gene expression data, and (3) SG-expressed genes from the BDGP database. The three-way overlap to identify known SG-expressed genes activated by Rib produced Sema-5c as the single likely candidate. Similar analysis for the identification of SG-expressed genes repressed by Rib resulted in qtc and CG5953 as likely candidates. Indeed, we confirmed the directionality of regulation of all three Rib targets by in situ hybridization analyses in rib mutants. Sema-5c is a transmembrane protein belonging to the semaphorin family of growth cone guidance molecules implicated in neural morphogenesis (Khare et al., 2000). Our in situ analysis of embryos from stages 12–14, when vigorous tube elongation occurs, shows Sema-5c expression in the WT SG, which is downregulated in rib mutants. This finding is consistent with the microarray gene expression analysis, but is not consistent with the qRT-PCR results (Fig. 4E; Table 5). qtc was among the candidates repressed by Rib (three-way overlap), qtc has been implicated in the regulation of normal male sexual behavior (Gaines et al., 2000). Although its mechanism of function is unknown, portions of the predicted Qtc protein have limited sequence identity to a large family of myosin and myosin-like proteins, all of which contain α-helical coiled coil domains with common structural features (Lupsas, 1996). The other candidate repressed by Rib (three-way overlap) is CG5953, which encodes a MADF domain containing protein (FlyBase). MADF (Myb/SANT-like domain in Adf-1) domain is composed of an 80-amino acid module that directs sequence specific DNA binding to sites with multiple tri-nucleotide repeats (Interpro). Based on the examples of proteins containing MADF domain (Adf-1 and Dip3) functioning as transcription factors (Cutler et al., 1998; England et al., 1992), we presume that CG5953 encodes for a transcription factor as well. Rib-dependent downstream transcription factors could be responsible for the large number of genes whose expression changes in rib mutants but that do not contain Rib binding sites. Indeed, although Rib occupancy was observed near the crb promoter in both ChIP-Seq datasets, this result fell out of our analysis due to a lack of signal overlap using the different drivers (Supplemental Fig. 1Y). The lack of overlap may simply be a result of the stringent ChIP-seq peak calling thresholds we used for identifying high confidence direct Rib targets. Alternatively, this could reflect a less significant role for Rib in direct SG regulation of crb, opening up the possibility that genes such as CG5953 or hairy, a bHLH transcription factor previously shown to repress SG gene expression (Myat and Andrew, 2002), could be involved. We also found no change in crb expression by microarray. This finding suggests that the microarray of whole embryos (stages 11–16) may not be sensitive enough to detect the stage-specific reduction in crb transcripts previously observed in the SG and trachea by in situ and by qRT-PCR of stage 12 embryos (Kerman et al., 2008). In general, our experience indicates that in situ analysis is a much more sensitive measure of tissue-specific changes in gene expression, especially for targets expressed in multiple tissues under the control of a variety of regulators (Maruyama et al., 2011).

We also examined the expression levels of mew, which encodes for the integrin alpha subunit and functions in cell adhesion to the extracellular matrix to facilitate migration of the SG and other tissues (Bradley et al., 2003; Brown et al., 2000). Although the whole embryo mew transcript levels were not significantly changed in the rib mutants with microarray gene expression analysis, we chose to examine its levels in the SG because of a strong ChIP-seq Rib binding peak in the proximity of the gene. Our in situ analysis showed decreased expression of mew transcripts in the mutants compared to WT SGs. In addition to mew, we examined the SG expression of Tre1, which encodes a G-protein coupled receptor involved in cell migration (Kunwar et al., 2003). The Tre1 transcript levels were downregulated in rib mutants based on the microarray gene expression analysis, and our in situ analysis confirmed these results, suggesting that Tre1 is a direct target of Rib in the SG. The results that mew and Tre1 are activated by Rib suggest that Rib activates genes known or presumed to be involved in SG migration. Overall, the known functions of target genes bound and regulated by Rib in whole embryos and in the SG are consistent with the defects in cell volume observed in the rib mutants. They are also consistent with a more direct role for Rib in morphogenesis, including a role in cell migration.
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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.10.016.

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