

The Garz Sec7 domain guanine nucleotide exchange factor for Arf regulates salivary gland development in *Drosophila*

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Surface delivery of proteins involved in cell-cell and cell-matrix interactions in cultured mammalian cells requires the GBF1 guanine nucleotide exchange factor. However, the role of GBF1 in delivery of adhesion proteins during organogenesis in intact animals has not been characterized. Here, we report the function of the fly GBF1 homolog, Gartzweg (Garz) in the development of the salivary gland in *Drosophila melanogaster*. We used the GAL4/UAS system to selectively deplete Garz from salivary gland cells. We show that depletion of Garz disrupts the secretory pathway as evidenced by the collapse of Golgi-localized Lava lamp (Lva) and the TGN-localized γ subunit of the clathrin-adaptor protein complex (AP-1). Additionally, Garz depletion inhibits trafficking of cell-cell adhesion proteins cadherin (DE-cad) and Flamingo to the cell surface. Disregulation of trafficking correlates with mistargeting of the tumor suppressor protein Discs large involved in epithelial polarity determination. Garz-depleted salivary cells are smaller and lack well-defined plasma membrane domains. Garz depletion also inhibits normal elongation and positioning of epithelial cells, resulting in a disorganized salivary gland that lacks a well defined luminal duct. Our findings suggest that Garz is essential for establishment of epithelial structures and demonstrate an absolute requirement for Garz during *Drosophila* development.

Introduction

Developmental processes in multicellular organisms are coordinated through surface adhesion and signaling molecules. The availability of these components on cell surfaces is dependent on efficient trafficking from the site of synthesis at the endoplasmic reticulum (ER) to the plasma membrane (PM). One of the key events required for directional traffic is protein sorting. Cargo sorting requires formation of a molecular coat on the cytoplasmic surface of the membrane that sequesters cargo proteins within a membrane patch to form a transport vesicle. Coats functioning within the secretory pathway include the heptameric COPI coat and clathrin coats assembled from clathrin triskelions and adaptor proteins.¹⁻⁵ Both types of coats are recruited to membranes by ADP ribosylation factors (Arfs), members of the Ras family of small GTPases.⁶ Coat recruitment requires activated Arfs. Arfs cycle between an inactive GDP-bound form and an active GTP-bound form and the exchange of GDP for GTP in cells is catalyzed by Sec7 domain-containing guanine nucleotide exchange factors (GEFs).^{7,8} Although GEFs have been shown to regulate coating events and are critical regulators of intracellular trafficking, the role that GEFs play in developmental events is largely unknown.

The mammalian GEF GBF1 is essential for Golgi complex biogenesis. Inactivation of GBF1 by RNAi-mediated depletion,

expression of a dominant negative form of GBF1 or treatment with the GBF1-specific inhibitor Golgicide collapses the secretory pathway.⁹⁻¹³ GBF1 is essential for trafficking of cargoes between the ER and the Golgi and GBF1 depletion in cultured cells disrupts trafficking of surface adhesion proteins such as E-selectin ligand I (ESL-1), a broadly expressed protein implicated in leukocyte-endothelial cell interactions.¹⁴ GBF1 depletion also inhibits migration of glioma cells.¹⁰ The importance of GBF1 in mammalian cell adhesion and motility suggested that GBF1 may regulate cell adhesion and migration during morphogenetic changes that sculpt organs in multicellular organisms.

We explored the role of GBF1-regulated pathways in the model organism *Drosophila melanogaster*, focusing on development of the salivary gland. Morphogenesis of the salivary gland requires coordinated cell shape changes, cell intercalations and direct cell migration of the ~135 ectodermal salivary gland precursor cells.^{15,16} Salivary gland cells require extensive changes in adhesion properties to internalize and form a tube that then extends posteriorly by extensive cell-cell and cell-matrix interactions. Flies with defects in genes encoding various adhesion molecules, such as integrins or DE-cadherin exhibit defects in tube elongation and abnormal cellular shape.¹⁵⁻²¹ Defects in adhesion result in small, poorly organized salivary glands.²² We posited that similar phenotypes might be apparent if Garz

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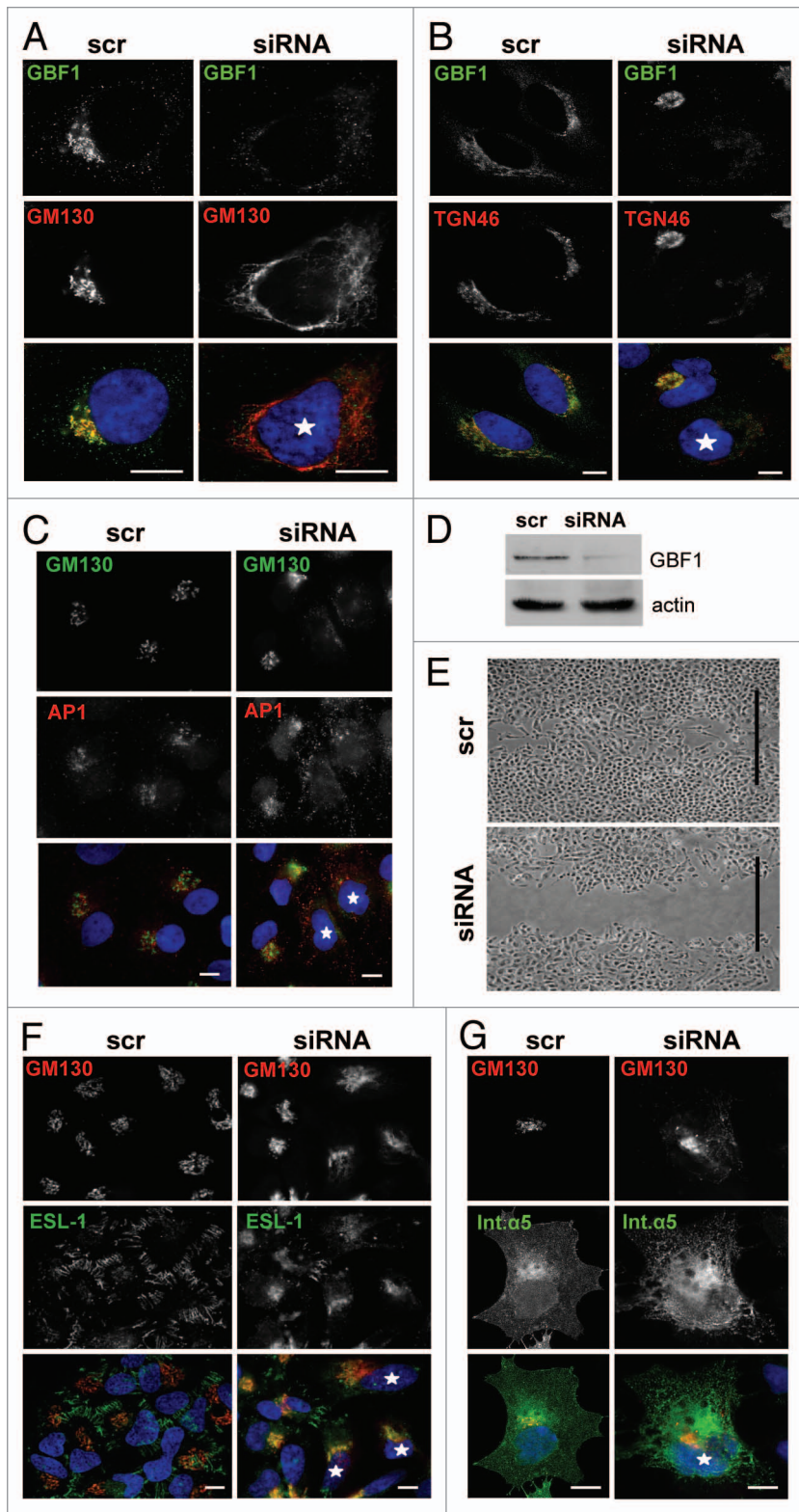


Figure 1. Effects of GBF1 depletion in mammalian cells. (A–C and F) HeLa cells were transfected with scrambled (scr) or anti-GBF1 siRNA (siRNA) and after 72 h were either processed for IF with the indicated antibodies. (D) HeLa cells were transfected with scrambled (scr) or anti-GBF1 siRNA (siRNA) and after 72 h were lysed and cell lysates processed by SDS-PAGE and immunoblotted to assess depletion of GBF1. (E) HeLa cells were transfected with scrambled (scr) or anti-GBF1 siRNA (siRNA) and after 72 h cells were removed from the middle of the plate by scraping and the wound analyzed 12 h later. (G) HeLa cells were transfected with GFP-tagged integrin- α 5 for 12 h following transfection with either scrambled siRNA (scr) or anti-GBF1 siRNA (siRNA) for 48 h. Cells were then processed for IF with the indicated antibodies. Examples of depleted cells are marked with asterisks. Nuclei were stained with Hoechst. Bars in (E) indicate the site of the wound. Bars, 10 μ m. GBF1 depletion is efficient (D) and causes tubulation of the cis-Golgi (A), disrupts the TGN (B), inhibits membrane recruitment of the AP-1 clathrin adaptor (C), inhibits trafficking of the adhesion proteins ESL-1 (F) and integrin- α 5 (G) and inhibits cell migration (E).

pathway, inhibits trafficking of adhesion molecules DE-cadherin (DE-cad) and Flamingo to the cell surface, disrupts the localization of the tumor suppressor Discs large (Dlg) involved in polarity determination and causes a dramatic disorganization of the salivary gland. Our results indicate that Garz is essential for developmental events occurring during organ morphogenesis and establishes a critical role for this GEF in tissue and organismal homeostasis.

Results

Mammalian GBF1 is required for intact secretory pathway, trafficking of adhesion proteins and cell motility. To provide a baseline for comparing GBF1 function in mammalian and *Drosophila* systems, we first assessed cellular effects of GBF1 depletion in mammalian cells. GBF1 was depleted from HeLa cells by siRNA, as confirmed by immunoblotting (Fig. 1D). Quantitation of similar immunoblots indicates that we routinely deplete 80 to 90% of cellular GBF1. GBF1 depletion caused extensive tubulation of the cis-Golgi, as evidenced by relocation of the GM130 golgi marker from the typical perinuclear Golgi ribbon into a network of tubular elements (Fig. 1A), in agreement with previ-

ous reports in references 9–11. We marked GBF1-depleted cells with asterisks to facilitate their identification. We also chose to include panels showing GBF1-containing and GBF1-depleted cells. We stress that we searched for fields containing undepleted cells among the vast majority of depleted cells. We elected to search for such fields to clearly show the difference in localization

plays a role in trafficking proteins required for salivary gland development. We used the GAL4/UAS system and RNAi to selectively deplete the fly homolog of mammalian GBF1, Gartzweg (Garz), from salivary glands.^{23,24} We document that depletion of Garz from salivary gland cells collapses the secretory

pathway, inhibits trafficking of adhesion molecules DE-cadherin (DE-cad) and Flamingo to the cell surface, disrupts the localization of the tumor suppressor Discs large (Dlg) involved in polarity determination and causes a dramatic disorganization of the salivary gland. Our results indicate that Garz is essential for developmental events occurring during organ morphogenesis and establishes a critical role for this GEF in tissue and organismal homeostasis.

of markers in GBF1-containing and GBF1-depleted cells. The extent of tubulation varies in depleted cells, but all cells show disruption of the usual peri-nuclear GM130 pattern. GBF1 depletion also disrupted the architecture of the TGN, as shown by the dispersal of the TGN46 marker from a perinuclear ribbon in control cells to peripheral punctate elements (Fig. 1B).

GBF1 mediates recruitment of the COPI coat to early secretory compartments and tubulation of GM130 correlates with the dissociation of COPI from membranes in GBF1-depleted cells.^{10,11} The disruption of the TGN suggested that GBF1 depletion also may affect membrane recruitment of TGN coats.³¹ Indeed, localization of the γ subunit of the AP-1 clathrin adaptor known to function at the TGN³² is altered in GBF1-depleted cells (Fig. 1C). In these experiments, GBF1-depleted cells are detected by tubulated GM130. Indirect assessment of GBF1 depletion was required because of antibody availability and/or compatibility. In control cells, AP-1 localizes to the TGN, but AP-1 is largely dispersed in GBF1-depleted cells, in agreement with a previous report in reference 11. Thus, in mammalian cells GBF1 appears to control the architecture of early (cis-Golgi) and late (TGN) secretory compartments.

To probe the role of GBF1 in trafficking of transmembrane proteins involved in cell-cell and cell-matrix interactions, we assessed the localization of E-selectin ligand 1 (ESL-1) and integrin- $\alpha 5$. In control HeLa cells ESL-1 localized to thin PM projections, where it participates in cell-cell interactions (Fig. 1F). In cells depleted of GBF1 (detected here by tubulated GM130), ESL-1 was not concentrated on cell surface and, instead, much of ESL-1 was retained inside cells, in agreement with our previous report in reference 10. GBF1 depletion also inhibited PM delivery of integrin- $\alpha 5$. In initial experiments, we analyzed PM localization of endogenous integrin- $\alpha 5$ after depleting GBF1 for 72 h and did not observe detectable differences between control and GBF1-depleted cells (not shown). As this was likely due to a relatively slow turnover of integrin- $\alpha 5$, we assessed trafficking of integrin- $\alpha 5$ expressed exogenously after GBF1 depletion was initiated. HeLa cells were transfected with anti-GBF1 siRNA for 48 h and then transfected with GFP-tagged integrin- $\alpha 5$ and analyzed after 24 h. In control cells, integrin- $\alpha 5$ localized in the perinuclear region and was detected in a punctate distribution on the PM (Fig. 1G). In contrast, cells depleted of GBF1 lack surface integrin- $\alpha 5$, with some integrin- $\alpha 5$ appearing retained in the ER. Regulating integrin trafficking represents a novel function for GBF1.

Both ESL-1 and integrin- $\alpha 5$ are implicated in cell motility, suggesting that GBF1-depleted cells might be compromised in movement. A cell-wounding assay was used to show that control cells actively migrate towards opposite edges of the wound and largely close the wound in 12 h (Fig. 1E). In contrast, GBF1-depleted cells did not close the wound area, documenting a novel requirement for GBF1 in wound closure. Thus, GBF1 is essential for delivery of adhesion proteins involved in cell motility and for migration of mammalian cells in culture.

Garz depletion in salivary gland cells disrupts the secretory pathway. Mammalian cells contain three Arf-GEFs (GBF1, BIG1 and BIG2) that appear to regulate membrane trafficking.⁷

Drosophila has a homolog of GBF1 called Garz and a homolog of BIG1 called Sec71, but appears to lack a BIG2 homolog (FlyBase). The role of Garz in maintaining the secretory pathway in larval salivary gland cells was assessed by generating transgenic flies carrying the salivary gland GAL4 driver (OK6) and UAS>RNAi^{Garz}. Depletion of Garz in transgenic animals was confirmed by RT-PCR of salivary glands from wild-type and OK6>GAL4/UAS>RNAi^{Garz} flies. The *GARZ* gene gives rise to two transcripts of 5,317 bp (short form A) and 6,278 bp (long form B), and both are detected in control salivary glands (Fig. 2A). Both transcripts are reduced by ~80% in salivary glands from flies expressing Garz RNAi. The actin control shows equivalent amount of actin mRNA in control and Garz-depleted salivary glands.

The effect of depleting Garz on the integrity of the secretory pathway in salivary cells was assessed by the localization of the *Drosophila* golgin, Lva and the γ subunit of the AP-1 clathrin adaptor. The Golgi and TGN in *Drosophila* cells don't form a peri-nuclear ribbon and instead are organized into separate mini-stacks dispersed throughout the cell.³³ Like mammalian Golgi ribbons, each Golgi mini-stack in *Drosophila* cells is polarized, with cis/medial Golgi elements positioned adjacent to but separate from the TGN. In control salivary cells Lva localizes to the cis-Golgi^{34,35} and shows a characteristic punctate pattern of Golgi elements scattered throughout the cells (Fig. 2B). AP-1 is a TGN marker³⁶ and in control salivary cells localizes to structures adjacent to, but distinct from, Lva-marked Golgi bodies (Fig. 2B). In Garz-depleted salivary cells, the Golgi and the TGN show dramatic collapse, with Lva and AP-1 detected in diffuse patterns and in few punctate structures that lack the clear definition of Golgi mini-stacks (Fig. 2B). Thus, Garz in *Drosophila* regulates the secretory pathway in a manner analogous to the function of GBF1 in mammalian cells.

Garz depletion in the salivary gland inhibits trafficking of adhesion molecules and disrupts gland architecture. The role of Garz in the trafficking of adhesion proteins was assessed by localization of two members of the *Drosophila* cadherin family, DE-cad and Flamingo. DE-cad is homologous to vertebrate classic cadherins (E-cadherins) and is concentrated in adherens junctions where it associates with α - and β -catenin.^{37,38} DE-cad is required for epithelial polarization and cell migration.^{39,40} Flamingo is a 7-pass transmembrane domain receptor that localizes at cell-cell boundaries.⁴¹⁻⁴⁴ Flamingo directs establishment of tissue polarity in a number of organs, and this function is linked to the extracellular domains that mediate cell-cell adhesion. Defects in distribution of Flamingo disrupt planar cell polarity, possibly by inability of cells to undergo normal shape changes.^{45,46} In control salivary glands, DE-cad and Flamingo accumulate at cell-cell junctions, resulting in a hexagonal "chicken-wire" pattern of cell outlines (Fig. 3A). In contrast, in Garz-depleted cells, DE-cad and Flamingo are largely absent from the PM. In some Garz-depleted cells, DE-cad is seen in internal punctate structures (arrowheads). Flamingo is not detected in recognizable intracellular structures.

Another protein that functions in the establishment of epithelial cell polarity is the tumor suppressor Discs large (Dlg).^{47,48}

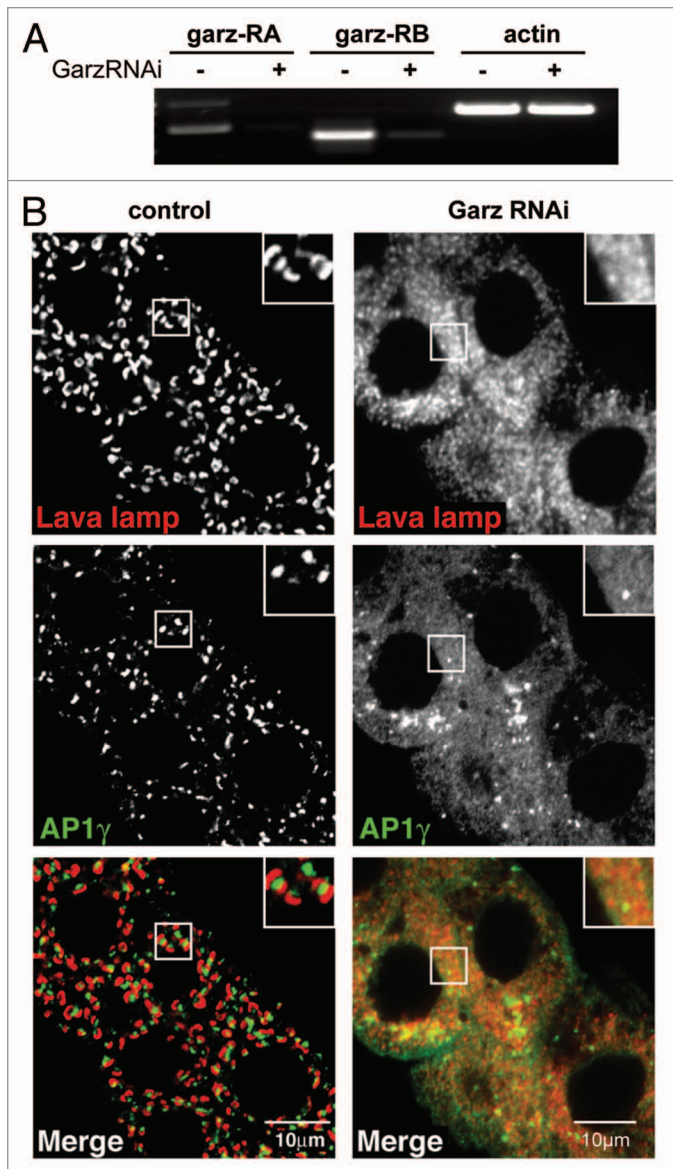


Figure 2. Garz depletion collapses the secretory pathway in *Drosophila* salivary gland cells. Salivary glands were dissected from control or Garz-depleted third instar (mid-L3) larva and either subjected to RT-PCR to detect two splicing transcripts of *garz* (transcript A: 5,317 bp and transcript B: 6,278 bp) and an actin transcript (A) or processed for IF with the indicated antibodies (B). Bar in (B) 10 μ m. Insets show enlarged areas of the original image marked by rectangles. Garz depletion is efficient (A) and disrupts the Golgi and TGN (B).

Dlg is a cytoplasmic protein that is recruited to septate junctions (homologous to tight junctions in vertebrates) through interactions with membrane proteins and actin-binding proteins⁴⁷ and is essential for formation of septate junctions during salivary gland maturation.^{48,49} In control salivary glands, Dlg localizes to lateral membranes (Fig. 3B). In contrast, in Garz-depleted salivary cells Dlg mislocalizes to apical regions of cells. This apical relocation is similar to that observed in salivary gland cells with disrupted AP-1 function⁵⁰ and is consistent with the finding that Garz depletion inhibits AP-1 recruitment to membranes (Fig. 2B).

To assess the role Garz plays in establishing the architecture of salivary glands, we examined localization of GFP-actin. In control salivary glands, GFP-actin marks basolateral and apical membranes of epithelial cells and outlines the lumen of the gland (Fig. 3C), in agreement with previous reports in references 51–53. The epithelial cells are columnar and elongated in the basal to apical direction, with a centrally localized nucleus. The nuclei in distinct cells appear in register. Control salivary glands are ~51 nm in width and ~69 nm in length (Table 1). In contrast, Garz-depleted glands are significantly smaller, ~36 nm in width and 34 nm in length and disorganized. GFP-actin outlines much smaller cells that are not columnar and appear haphazardly packed. GFP-actin doesn't appear to associate with basolateral membranes and instead is concentrated in patches in what should be the apical region of cells. The smaller size and disorganized architecture of Garz-depleted salivary glands suggests that Garz is essential to support cellular trafficking critical for morphogenic events that sculpt the salivary gland in *Drosophila*.

Discussion

We and others have shown previously that the Arf activator GBF1 is essential for the establishment of functional secretory pathway and for trafficking of proteins in mammalian cells in culture. Herein, we document the role of GBF1 homolog Garz in the maintenance of the secretory pathway and in trafficking of proteins in intact *Drosophila*, and show that GBF1 is required for development of salivary glands in the fly.

GBF1 is critical for biogenesis of the Golgi ribbon in mammalian cells^{10,13,25,54-56} and for recruitment of the COPI coat that mediates protein traffic at the ER-Golgi interface and of the AP-1 and GGA adaptors that function at the TGN.^{10,11,13,25,57} The architecture of the Golgi differs in mammalian and *Drosophila* cells and the Golgi ribbon found in mammalian cells is replaced by scattered but still polarized Golgi mini-stacks in *Drosophila* cells.^{58,59} Irrespective of this structural difference, we show that GBF1 depletion from mammalian cells and Garz depletion from salivary gland cells in living animals collapses the secretory pathway and dissociates the AP-1 coat. Thus, GBF1/Garz is required to maintain the cognate Golgi architecture and GBF1/Garz depletion causes a similar phenotype of Golgi collapse and coat dissociation.

GBF1 is essential for trafficking of proteins including adhesion proteins ESL-1 and integrin- α 5 from the ER to the cell surface.^{10,25} Herein, we document that Garz depletion inhibits surface delivery of adhesion proteins DE-cadherin and Flamingo in *Drosophila* salivary gland cells. Thus, GBF1/Garz appears to be required for trafficking of at least five transmembrane proteins (VSV-G,²⁵ ESL-1, integrin- α 5, DE-cadherin and Flamingo), suggesting a general effect on membrane trafficking rather than a cargo-specific effect. Furthermore, GBF1/Garz regulates cargo transit in mammalian and insect cells, despite the differences in Golgi morphology between the species.

The lack of surface delivery of adhesion proteins in GBF1-depleted mammalian cells correlates with decreased cell migration. This finding propelled us to investigate the consequences

of Garz depletion during development of salivary glands. The salivary gland of *Drosophila* is an excellent model system for studying morphological aspects of organ development.^{19,22} Once the number of primordial cells is specified, organ growth is achieved by an increase in size of individual salivary cells rather than continuing division. Development of the tubular organ is achieved predominantly by cell expansion, elongation of the tube and coordinate rearrangements of salivary cells to form a columnar epithelium.²⁰ All these processes are critically dependent on surface adhesion and signaling proteins. DE-cadherin has been shown to be critically important for epithelium formation and to facilitate cell migration and sorting processes that sculpt epithelial organs.^{60,61} Similarly, the atypical cadherin Flamingo has been shown to have a key role in epithelial morphogenesis.⁶²⁻⁶⁴ Based on the observed inhibition in DE-cadherin and Flamingo trafficking in Garz-depleted cells, we expected changes in salivary gland morphology. Indeed, we observed a significant decrease in salivary gland size in Garz-depleted animals. The decreased gland size appears to be caused by the smaller size of individual cells, as the number of cells does not appear to be altered in Garz-depleted glands. This suggests that differentiation events that generate salivary gland primordia occur in the context of our experiments. Most likely, OK6/GAL4-induced expression of the Garz RNAi in the salivary gland occurs after specification of the salivary gland primordial cells, at a time when the salivary gland cells undergo elongation and changes in cell shape. Garz depletion causes significant disruption in salivary gland development by inhibiting cell expansion and rearrangements and preventing the formation of a columnar epithelium arranged into an elongated tube. Our findings in Garz-depleted animals are consistent with results from a targeted gain-of-function screen in which overexpression of Garz at early stages of gland development resulted in the formation of abnormal hooked salivary glands.⁶⁵

The observed disorganization of the Garz-depleted salivary glands correlates with disruption in the localization of Dlg and subcortical GFP-actin, suggesting disturbance in the establishment or maintenance of cell polarity. The Garz-depletion phenotype is similar to that caused by mutations in Nak, a kinase that regulates phosphorylation of AP complexes.⁵⁰ Nak mutants show disruption of the lateral Dlg localization and have severely retarded growth of salivary glands.

Dlg interacts with actin-binding proteins and this link to the actin cytoskeleton may allow Dlg to mediate cell shape changes, leading to cellular extensions or reinforcement of cell-cell contacts.^{48,49} The disorganization of Dlg in Garz-depleted organs correlates with disorganization of the actin cytoskeleton and it is likely that both contribute to lack of correct developmental patterning. Dlg interacts genetically with the Exo84 component of the exocyst⁶⁶ and thereby may regulate the polarized insertion of newly synthesized membranes that contribute to polarized expansion of cell membranes. Furthermore, the exocyst regulates DE-cadherin traffic from recycling endosomes to the plasma membrane,⁶⁷ suggesting that disruption of exocyst function may contribute to decreased levels of surface DE-cadherin. The reduced size of cells in Garz-depleted cells may reflect the

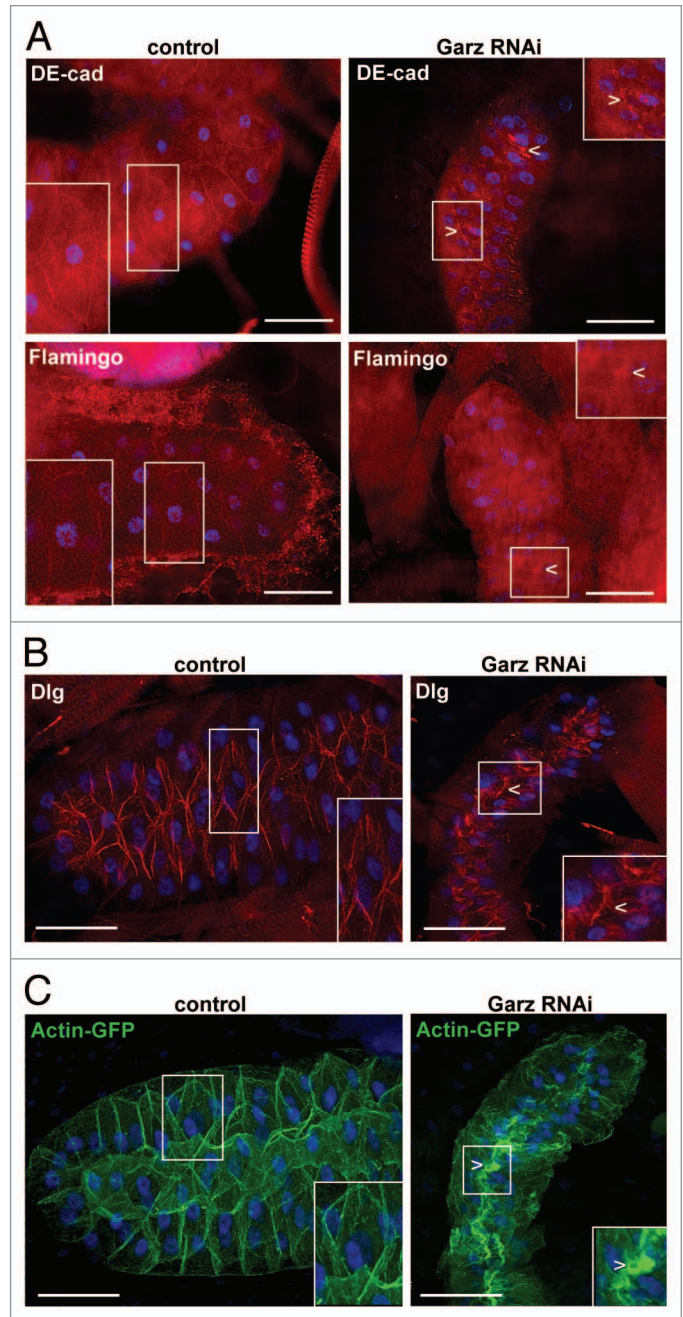


Figure 3. Garz depletion inhibits surface delivery of adhesion proteins and disrupts development of salivary glands in *Drosophila*. Salivary glands were dissected from control or Garz-depleted third instar (late L-3) larva, processed for IF with the indicated antibodies and the nuclei were stained with Hoechst (A and B). Salivary glands from control or Garz-depleted third instar larva expressing actin-GFP transgene were stained with Hoechst and imaged (C). Bars, 100 μm. Insets in (A) show enlarged areas. Garz depletion inhibits trafficking of DE-cad and Flamingo adhesion proteins to cell surface (A), disrupts localization of Dlg (B) and causes disruption in salivary gland development (C).

lack of membrane insertion in addition to reduced trafficking of adhesion proteins.

Our findings demonstrate a role for Garz in development of the *Drosophila* salivary gland. Since developmental mechanisms

Table 1. Dimensions of control and Garz-depleted salivary glands

	Garz RNAi cells late L3		Control late L3	
	Width	Length	Width	Length
Average	35.5	34.3	50.8	68.6
Stdev	5.5	5.3	11.0	10.3
	n = 19		n = 19	
	Student's two-tailed t-test (Garz vs. Control)			
	Width	Length		
	4.7074E-05	3.168E-11		

appear conserved, Garz is likely essential for other developmental events in the fly. Indeed, silencing Garz within photoreceptors R1-R7 causes their degeneration (not shown), suggesting that many developmental events in the fly require Garz. Garz and GBF1 have orthologs/paralogs in all organisms.⁷ Considering the conservation of cellular functions observed for the mammalian GBF1 and the *Drosophila* Garz, it is likely that GBF1 is a key regulator of pathways critical for development in mammals. Our work sets the paradigm for such analyses.

Experimental Procedures

Antibodies. Rabbit polyclonal anti-GBF1 antibodies have been described in reference 25. Rabbit polyclonal antibodies against ESL-1 were from M.K. Wilde (University of Muenster, Muenster, Germany). Mouse anti-AP-1 γ antibodies were from Dr. Roland Le Borgne (CNRS, Rennes Cedex, France) and rabbit anti-Lva antibody was from Dr. John Sisson (The University of Texas, Austin, TX). Mouse anti-Dlg antibodies were developed by Dan Woods and Peter Bryant, mouse anti-Flamingo and rat anti-DE-cad antibodies were developed by Tadashi Uemura; all were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The following antibodies were commercially obtained: polyclonal anti-GFP (Abcam, Ab290), monoclonal anti-GM130, polyclonal anti-actin and monoclonal anti-AP-1 antibodies (Sigma-Aldrich, G7295, A5060, A9856), sheep anti-TGN46 antibodies (Serotec, AHP500G). Secondary antibodies conjugated with HRP, Alexa 488, Alexa-568 or Alexa 594 were from Molecular Probes (A11034, A12380, A11032).

Fly stocks. *P{GawB}ABI-GAL4* and *P{GawB}OK6* flies (obtained from the Bloomington *Drosophila* stock center) were crossed to UAS-RNAi^{Garz} flies to deplete Garz in the salivary gland. The OK6 driver is part of a promoter for the *Rappap1* gene that mediates GAL4 expression in both motor neurons (*Rappap1* is expressed at high levels in motor neurons) and salivary cells, most likely because this construct contains a fortuitous salivary gland enhancer element.²⁶ $P\{w[+m^*] = GAL4-OK6\}$, $P\{UAS GFP-actin, w[+]\}$ flies were generated by Dr. Marques (UAB) and crossed to UAS-RNAi^{Garz} to deplete Garz in salivary glands expressing GFP-tagged actin. *P{GawB}ABI-Gal4* is an enhancer trap element that displays constitutive expression of GAL4 in larval salivary glands.²⁷ *P{GawB}ABI-Gal4* was crossed to UAS-RNAi^{Garz} to analyze AP-1 and Lva distribution.

Mammalian cell culture, transfection and depletion. HeLa cells were grown as in reference 25. Hs_GBF1_5 siRNA oligos directed against human GBF1 (5'-CAC AAG GTT ACC TCA GTT TAA-3') were purchased from Qiagen (SI03158750). Nontargeting siRNA were designed and synthesized as annealed primers by Ambion (4611). HeLa cells were transfected with siRNA using siLentFect lipid (BioRad Laboratories, 170-3361) according to manufacturer's instructions. Cells were processed for IF, immunoblotting or subjected to a scratch assay 72 h after depletion. In some experiments, cells were transfected with siRNA for 48 h and then transfected with GFP-tagged integrin- $\alpha 5$ using the Mirus TransIT-LT1 reagent (Mirus Bio Corporation, MIR2300) according to manufacturer's instructions for additional 24 h. Cells were then processed for immunofluorescence (IF).

Scratch assays were performed by removing cells with a small pipettor tip from a middle of a confluent plate and observing the scratched region 12 h later.

Microscopy. HeLa cells were processed for immunofluorescence (IF) as in reference 25. Salivary glands from third instar larvae were dissected in PBS (pH 7.4), and fixed for 20 min on ice in PLP (4% paraformaldehyde, 0.01 M sodium meta-periodate, 0.075 lysine, 0.035 phosphate buffer, pH 7.4). Fixed salivary glands were then washed once in PBS (pH 7.4) and permeabilized in PBST (PBS + 0.1% Triton X-100). Primary antibody incubation was performed overnight at 4°C in PBST with 5% normal goat serum. Salivary glands were mounted in PPD (0.1x PBS, 90% glycerol, 1 mg/ml *p*-phenylenediamine).

Fluorescence was visualized with a Leitz Orthoplan microscope with epifluorescence and Hoffman Modulation Contrast optics from Chroma Technology. Images were captured with a CCD high-resolution camera from Roper Scientific equipped with a camera/computer interface. Images were analyzed with a power Mac using IPLab Spectrum software (Scanalytics). Confocal imaging was performed on Leica DMRXE upright, epifluorescence-Nomarski microscope outfitted with Leica TCS SP2 laser confocal optics (Leica). Optical sections through the Z axis were generated using a computer-controlled focus step motor. Flattened projections of image stacks and 3D renderings were prepared using proprietary confocal imaging software from Leica. Fluorescence images of Lva and AP-1 were acquired on a Zeiss LSM510 inverted laser scanning confocal microscope equipped with LSM objectives (63x-Plan-APOCHROMAT NA 1.4) and LSM510 software. All images were further processed for brightness and contrast levels using Adobe Photoshop CS2.

Garz depletion. A transgene promoting RNAi-mediated Garz depletion was generated according to methodology described in Kalidas and Smith.²⁸ The construct consists of a hairpin with half of the DNA derived from a cDNA, and the other half from genomic DNA (and contains intronic sequences). The intron is spliced out during the processing of the RNA to generate a hairpin double-stranded RNA that is completely complementary to each other. Primers for generating the hairpin fragment, 5'-TCC TAA ATG CCG AGC TTG ATC C-3' (in exon 4) and 5' GTG ATA CCC TTC CGG CTA CG-3' (in exon 9), were used to amplify cDNA and genomic DNA. These were cloned into the pUAST

vector²⁹ and the construct was injected into embryos using standard methods to generate transgenic animals (UAS>RNAi^{Garz}). The entire coding sequence targeted by the RNAi construct is 3,143 bp, of which 2,988 bp overlaps with transcripts A and B.

SDS-PAGE and immunoblotting. HeLa cells silenced for 72 h with scrambled or anti-GBF1 siRNA were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0) supplemented with complete protease inhibitors from Roche (11873580001). Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters and immunoblotted as in reference 30.

RT-PCR. Salivary glands were dissected from ten third instar larvae from control OK6>GAL4 and transgenic OK6>GAL4; UAS>RNAi^{Garz} animals. Total RNA was extracted using

AurumTM Total RNA Mini Kit from BioRad (732-6820). Reverse transcriptase reaction and Single strand cDNA amplifications with specific primers for two spliced transcripts of Garz and actin were performed according to manufacturer's protocol utilizing OneStep RT-PCR Kit from Qiagen (210210). Samples were analyzed on a 1% agarose gel.

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