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Armbruster, Kristina; Luschnig, Stefan

Abstract: Protein trafficking through the secretory pathway plays a key role in epithelial organ development and function. The expansion of tracheal tubes in Drosophila depends on trafficking of coatomer protein complex I (COPI)-coated vesicles between the Golgi complex and the endoplasmic reticulum (ER). However, it is not clear how this pathway is regulated. Here we describe an essential function of the Sec7 domain guanine nucleotide exchange factor (GEF) gartenzwerg (garz) in epithelial tube morphogenesis and protein secretion. garz is essential for the recruitment of COPI components and for normal Golgi organization. A GFP–Garz fusion protein is distributed in the cytoplasm and accumulates at the cis-Golgi. Localization to the Golgi requires the C-terminal part of Garz. Conversely, blocking the GDP–GTP nucleotide exchange reaction leads to constitutive Golgi localization, suggesting that Garz cycles in a GEF-activity-dependent manner between cytoplasmic and Golgi-membrane-localized pools. The related human ARF-GEF protein GBF1 can substitute for garz function in Drosophila tracheal cells, indicating that the relevant functions of these proteins are conserved. We show that garz interacts genetically with the ARF1 homolog ARF79F and with the ARF1-GAP homolog Gap69C, thus placing garz in a regulatory circuit that controls COPI trafficking in Drosophila. Interestingly, overexpression of garz causes accumulation of secreted proteins in the ER, suggesting that excessive garz activity leads to increased retrograde trafficking. Thus, garz might regulate epithelial tube morphogenesis and secretion by controlling the rate of trafficking of COPI vesicles.

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The *Drosophila* Sec7 domain guanine nucleotide exchange factor protein Gartenzwerg localizes at the cis-Golgi and is essential for epithelial tube expansion

Kristina Armbruster and Stefan Luschnig*
Institute of Molecular Life Sciences, University of Zurich, CH-8057 Zurich, Switzerland

*Author for correspondence (stefan.luschnig@imls.uzh.ch)

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Summary

Protein trafficking through the secretory pathway plays a key role in epithelial organ development and function. The expansion of tracheal tubes in *Drosophila* depends on trafficking of coatomer protein complex I (COPI)-coated vesicles between the Golgi complex and the endoplasmic reticulum (ER). However, it is not clear how this pathway is regulated. Here we describe an essential function of the Sec7 domain guanine nucleotide exchange factor (GEF) *gartenzwerg* (*garz*) in epithelial tube morphogenesis and protein secretion. *garz* is essential for the recruitment of COPI components and for normal Golgi organization. A GFP–*Garz* fusion protein is distributed in the cytoplasm and accumulates at the cis-Golgi. Localization to the Golgi requires the C-terminal part of *Garz*. Conversely, blocking the GDP–GTP nucleotide exchange reaction leads to constitutive Golgi localization, suggesting that *Garz* cycles in a GEF-activity-dependent manner between cytoplasmic and Golgi-membrane-localized pools. The related human ARF-GEF protein GBF1 can substitute for *garz* function in *Drosophila* tracheal cells, indicating that the relevant functions of these proteins are conserved. We show that *garz* interacts genetically with the ARF1 homolog ARF*79F* and with the ARF1-GAP homolog Gap69C, thus placing *garz* in a regulatory circuit that controls COPI trafficking in *Drosophila*. Interestingly, overexpression of *garz* causes accumulation of secreted proteins in the ER, suggesting that excessive *garz* activity leads to increased retrograde trafficking. Thus, *garz* might regulate epithelial tube morphogenesis and secretion by controlling the rate of trafficking of COPI vesicles.

Key words: Golgi, COPI, ARF, ARF-GEF, GBF1, *Gartenzwerg*, *Drosophila melanogaster*, Tracheal system, Epithelial tube, Membrane traffic

Introduction

The dimensions and shapes of epithelial tubules in many organs, such as vertebrate kidneys, lungs and vascular systems, are precisely controlled to ensure normal organ function (Ghabrial et al., 2003; Lubarsky and Krasnow, 2003; Baer et al., 2009). However, the cellular and molecular mechanisms underlying tube size control are not well understood. Recent work in various model systems highlights the importance of membrane and protein trafficking for the morphogenesis and function of epithelial tubes (Datta et al., 2011). Genetic studies of tracheal development in *Drosophila* revealed an important function of the secretory apparatus for the expansion and functional maturation of tubular epithelia. Tracheal tubes rapidly expand in diameter to attain their functional dimensions at the end of embryogenesis (Beitel and Krasnow, 2000). The diametric expansion (dilation) of the tracheae depends on the transport of proteins into the lumen (Tsarouhas et al., 2007). Although the precise roles of membrane trafficking and of luminal components in tube expansion are not yet clear, genetic mosaic analyses suggest that the diametric expansion of the tracheal lumen is driven by a cell-autonomous mechanism that promotes the enlargement of the apical cell surface (Förster et al., 2010). Thus far, all genes known to be required for tracheal lumen dilation encode components of the early secretory apparatus. These genes encode components of the anterograde (COPII) (Tsarouhas et al., 2007; Förster et al., 2010; Norum et al., 2010) and the retrograde (COPI) (Grieder et al., 2008; Jayaram et al., 2008) trafficking pathway between the ER and the Golgi complex. Proteins exit the ER in COPI-coated vesicles that subsequently fuse with the cis face of the Golgi (reviewed by Lee et al., 2004; Sato and Nakano, 2007; Spang, 2009). To maintain organelle homeostasis, the flow of membranes and proteins between the ER and Golgi has to be balanced. COPI-coated vesicles retrieve ER-resident proteins from the Golgi and the ER-Golgi intermediate compartment (ERGIC) and release them back into the ER (reviewed by Hsu and Yang, 2009). The formation of both COPII- and COPI-coated vesicles is regulated by G-proteins of the ARF/SAR superfamily (reviewed by Gillingham and Munro, 2007). COPI vesicle formation is initiated by the conversion of inactive GDP-bound ARF1 to its active GTP-bound form, as a result of the activity of a guanine nucleotide exchange factor (GEF) (Peyroche et al., 1996). Following the exchange reaction, ARF1-GTP undergoes a conformational change that allows stable ARF1 membrane anchorage (Franco et al., 1996; Antonny et al., 1997). This is thought to initiate the assembly of the coatamer complex on the Golgi membrane that leads to the budding of COPI-coated vesicles. ARF-GEF proteins share a central conserved Sec7 domain that facilitates ARF binding and catalyzes the nucleotide exchange reaction (Peyroche et al., 1996). In addition to the Sec7 domain, ARF-GEFs contain large conserved stretches whose functions are not well understood, but...
which are likely to be important for the localization and function of ARF-GEF proteins. Two subfamilies of so-called large ARF-GEFs are known, the Brefeldin-A-inhibited GEF (BIG) family proteins, which act on recycling endosomes at the trans-Golgi, and the Golgi-specific Brefeldin-A-resistant guanine nucleotide exchange factor 1 (GBF1) family proteins, which act at the cis-Golgi (reviewed by Anders and Jurgens, 2008; Zhao et al., 2002; Bui et al., 2009). GBF1 family ARF-GEFs have been characterized in yeast (Gea1/2) (Peyroche et al., 1996; Spang et al., 2001), mammalian cells (GBF1) (Claude et al., 1999; Kawamoto et al., 2002) and plants (GNOM, GNLI) (Richter et al., 2007). *Drosophila* contains a single GBF1 homolog called *gartenzwerg* (garz) (Kraut et al., 2001; Szul et al., 2011). However, *garz* loss-of-function mutations were not available and it was not clear which of the six annotated ARF-GEF homologs in *Drosophila* are involved in COPI vesicle formation at the Golgi. Despite a wealth of studies in cultured mammalian cells, very little is known about the function of GBF1 family ARF-GEFs during animal development. Here we describe the identification and functional characterization of *garz* mutations. We show that *garz* is an essential gene that is required for the morphogenesis of tubular epithelia during embryogenesis. Furthermore, *garz* is required for normal Golgi morphology and for localization of COPI components. Garz localizes at the cis-Golgi complex, and this localization depends on the C-terminal half of the Garz protein. We show that *garz* interacts genetically with the ARF1 homolog *ARF79F* and with the ARF1-GAP homolog *Gap69C*, thus placing *garz* in a regulatory circuit that controls COPI trafficking in *Drosophila*. The results of *garz* loss-of-function and gain-of-function experiments suggest that *garz* regulates tracheal tube expansion by modulating the rate of membrane flow between the Golgi and the ER.

**Results**

*angina* mutations affect tracheal tube expansion and protein secretion in epithelia

In a mutagenesis screen for genes involved in tracheal tube morphogenesis we isolated a lethal complementation group consisting of five alleles, which showed defects in tracheal lumen expansion (Fig. 1). Although early tracheal development was normal in the mutants, the lumen of the tracheal dorsal trunk (DT) failed to expand to its normal diameter and tracheal tubes did not fill with gas (Fig. 1B,F and data not shown). All five alleles showed similar tracheal defects and were embryonic lethal. We named the locus *angina* (*aga*) based on the narrow tracheal lumen phenotype. Besides the tracheae, other epithelial structures were defective in *aga* embryos. The salivary glands showed a narrower and bulbous lumen compared with those in the wild type (Fig. 1K,O). The larval cuticle was weakly pigmented and appeared transparent (Fig. 1L,P). Moreover, dorsal closure was delayed, although the dorsal epidermis eventually closed in most late-stage embryos. However, during dorsal closure, *aga* mutants showed fewer filopodia in the epidermal leading edge compared with wild-type embryos (supplementary material Fig. S1).

![Fig. 1. Effects of *angina* mutations on epithelial secretion and tubular organ development. (A–K,M–O)](image_url) Projections of confocal Z-stacks showing tracheal dorsal trunk (A–J,M,N) or salivary glands (K,O) in stage 16 wild-type (A–D, I–K) and *aga* mutant (E–H, M–O) embryos, respectively. (A–H) In wild-type embryos (A–D) Verm–RFP (magenta in A,E) is secreted into the tracheal lumen. Tracheal cells are marked with GFP (green in A,E). The membrane proteins SAS (magenta in C,G) and Fas III (green in C,G) localize to the apical and basolateral membrane, respectively. In *aga* mutant embryos (E–H) secretion of Verm–RFP (F) and SAS (H) is impaired and the proteins are partially retained intracellularly. Localization of Fas III (green in G) is unaffected in *aga* mutants. (L,J,M,N) Chitin (I) and Pio protein (J) accumulate in the tracheal lumen in stage 16 wild-type embryos. Deposition of chitin (M) and Pio (N) appear normal in *aga*/*aga* heterozygous (Df(2R)Exel6061) embryos (referred to as *aga*/*aga* embryos). Note that *aga* mutants show narrow tracheal lumina. (K,O) SAS staining (green) outlines the salivary gland lumen, while lateral cell membranes are marked by Fas III (magenta). Note that the salivary gland lumen in *aga* mutants (O) is narrower compared to the wild type (K). (L,P) Cuticle preparations of wild-type and *aga* mutant embryos. Wild-type embryos (L) show pigmented ventral denticle belts and head skeleton. *aga* mutants (P) show weak pigmentation of these structures. Scale bars: 10 μm (A–K,M–O), 100 μm (L,P).
In addition to the morphological defects, *aga* mutations affect protein secretion. Whereas Vermiform–RFP (Verm–RFP) protein accumulated in the tracheal lumen and was not detectable inside tracheal cells in wild-type stage 15 embryos (Fig. 1B), Verm–RFP was partially retained in tracheal cells of *aga* mutants (Fig. 1F). Similarly, endogenous Verm (Fig. 3A,B) and the related secreted protein Serpentine (Serp; data not shown), and the apical transmembrane (TM) protein Stranded at second (Sas; Fig. 1D,H) (Schonbaum et al., 1992) were partially retained in tracheal cells of *aga* mutants. By contrast, chitin was deposited normally in the tracheal lumen (Fig. 1M). Similarly, the localization of the zona pellucida protein Piopio (Pio) (Jazwinska et al., 2003) in the lumen of the tracheae and salivary glands appeared normal in *aga* mutants (Fig. 1N and data not shown). Moreover, despite the effects on protein secretion, *aga* mutations did not cause obvious defects in epithelial polarity, because apical (Crumb; data not shown) and basolateral (Fasciclin III; Fig. 1C,G,K,O, supplementary material Fig. S1) polarity markers localized correctly to the corresponding membrane domains in tracheal, salivary gland, and epidermal cells of *aga* mutants. Taken together, these findings indicate a requirement of *aga* for protein trafficking and morphogenesis in epithelial cells.

**angina alleles carry mutations in the ARF-GEF gene gartenzwerg**

We mapped the *aga* locus to the cytological interval 48F5–49A6 by non-complementation of Df(2R)Exel6061 (Parks et al., 2004). One gene in the interval, *gartenzwerg* (*gartz*/CG8487; FlyBase) (Kraut et al., 2001) encodes a GEF for ARF family G-proteins involved in vesicle trafficking (Fig. 2). *gartz* is a member of a conserved family of ARF-GEFs with homologs in humans (GBF1; 43% identity) (Claude et al., 1999), *Caenorhabditis elegans* (GBF1; 34% identity; WormBase), *Arabidopsis thaliana* (Gnom; 29% identity) (Busch et al., 1996) and *Saccharomyces cerevisiae* (Gea1, Gea2; 20% and 19% identity, respectively) (Spang et al., 2001). By sequencing the *gartz* coding region, we identified single point mutations in four of the five EMS-induced *aga* alleles (*gartz*_W982*, gartz*_R1044*, *gartz*_W1113, *gartz*_Q1427, Fig. 2A). Each of these mutations results in a premature termination codon located C-terminally to the Sec7 domain (Fig. 2B). Furthermore, a P-element (EP2028; Fig. 2A) (Bellen et al., 2004) inserted 298 bp upstream of the annotated transcription start site of *gartz* failed to complement all our *aga* alleles. EP2028 homozygous embryos showed tracheal defects resembling those of *aga* mutants (data not shown). Finally, tracheal-specific expression of Garz or GFP–Garz transgenes rescued the narrow tracheal lumen and the secretion defects of *aga* embryos (Fig. 3C,E, and data not shown). Together, these results indicate that the *aga* locus corresponds to the *gartz* gene. We therefore refer to *aga* as *gartz* from here on.

We found that in the EMS-induced *gartz* alleles the mutant mRNAs are detectable by semi-quantitative RT-PCR (supplementary material Fig. S2), suggesting that these mutant mRNAs, when translated, could give rise to C-terminally truncated proteins. We cannot exclude that such truncated Garz proteins could interfere with wild-type Garz or with other proteins in a dominant-negative fashion. However, tracheal...
phenotypes of garzR1044 and garzW1113 homozygous embryos were strictly recessive and were indistinguishable from the phenotypes of embryos carrying these mutations in trans to Df(2R)Exel6061 (Fig. 1E,F,M,N and data not shown). This suggests that the mutations, despite the presence of the catalytic Sec7 domain in the presumed truncated proteins, are strong or complete loss-of-function alleles.

garz is expressed in embryonic epithelia

Consistent with a function in epithelial morphogenesis and secretion, we found elevated levels of garz mRNA in epithelial tissues during embryogenesis. Zygotic garz transcripts were first detectable at stage 10 in the developing salivary gland (data not shown). Salivary gland signals became stronger during germband retraction and are maintained throughout embryogenesis (Fig. 2D,E). garz expression in tracheal cells was detectable from stage 11 onwards (Fig. 2D,E). From stage 14 on, garz transcripts were also detectable in the epidermis (Fig. 2E). Maternal garz mRNA is present in freshly laid eggs (Fig. 2C). Removal of garz function from the female germ line caused an early arrest in oogenesis, indicating that garz function is essential for cell survival or growth during oogenesis (data not shown). This finding further suggests that perduring maternal garz gene products mask a requirement of garz during early embryogenesis. Perdurance of maternal garz gene products is therefore likely to account for the late onset of the defects observed in zygotic garz mutants.

GFP–Garz localizes to the cis face of the Golgi complex

Drosophila Garz and its human homolog GBF1 were proposed to participate in COPI vesicle trafficking between the ER and the Golgi, as well as in endocytic uptake of glycosylphosphatidylinositol-anchored proteins (GPI-APs) (Mayor and Pagano, 2007; Gupta et al., 2009). To understand the role of garz in more detail, we examined the subcellular localization of a GFP–Garz fusion protein (Fig. 4). In transfected S2R+ cells, GFP–Garz was enriched at perinuclear punctate structures, whereas lower-level signals were distributed throughout the cytoplasm (Fig. 4A). Punctate signals overlapped with the Golgi marker p120 (Fig. 4B,C) (Stanley et al., 1997). The GFP–Garz signals also colocalized with the cis-Golgi matrix protein GM130 (Fig. 4D–F) (Sinka et al., 2008) and largely overlapped with the cis-Golgi-associated protein GMAP210 (Fig. 4G–I) (Friggi-Grelin et al., 2006). In epidermal cells in living embryos, GFP–Garz was enriched at punctate structures with lower levels distributed in the cytoplasm (Fig. 4J–L). The GFP–Garz punctae overlapped with the COPII component Sec31–RFP, which is enriched at ER exit sites (ERES) (Forster et al., 2010) (Fig. 4K,L). By contrast, Sec31–RFP punctae localized adjacent to, but not overlapping with the trans-Golgi marker galactosyltransferase (GalT)–GFP (Fig. 4M–O). Similarly, GalT–GFP signals were also adjacent to GMAP210 signals (Fig. 4P–R), indicating that our methods allow us to separate cis- and trans-Golgi compartments. We measured the distances between fluorescence intensity maxima of pairs of two markers at a given Golgi stack in epidermal cells (Fig. 4S). GFP–Garz maxima showed a mean distance of 0.08±0.054 μm (n=28) from Sec31–RFP maxima. By contrast, Sec31–RFP maxima were at a significantly greater distance from GalT–GFP maxima (mean distance 0.37±0.1 μm; n=33; P=0.0001) than from GFP–Garz maxima. Although the resolution limit did not allow us to distinguish between cis-Golgi and ERES, these results suggest that GFP–Garz is localized in closer proximity to the cis-face of the Golgi and to ERES than to the trans-Golgi compartment labeled by GalT–GFP. Whereas we observed that GFP–Garz accumulated adjacent to Golgi or ERES structures, we did not find GFP–Garz accumulations in proximity to the early endosomal marker Rab4–RFP (data not shown). Taken together, our data from S2 cells and embryos suggest that GFP–Garz accumulates specifically at the cis-Golgi.

The C-terminal portion of Garz is required for Golgi localization

All of our EMS-induced garz mutations are expected to give rise to C-terminally truncated proteins, but leave the catalytic Sec7 domain intact. Thus, these garz mutations might interfere with Golgi localization of the corresponding truncated proteins. To investigate this possibility, we analyzed the localization of wild-type and mutant GFP–Garz fusion proteins in tracheal cells and in transfected S2 cells (Fig. 5). GFP–Garz accumulated at the Golgi, whereas a fraction of the protein was distributed throughout the cytoplasm (Fig. 5A–D). By contrast, a C-terminally truncated protein corresponding to the garzW982 allele [GFP–Garz(W982)] failed to accumulate at the Golgi and was instead uniformly distributed in the cytoplasm of tracheal cells (Fig. 5E,F). Importantly, although GFP–Garz(W982) retains...
the catalytic Sec7 domain, expression of GFP–Garz(W982) failed to rescue the defects in garz mutants (data not shown) and expression in a wild-type background did not interfere with garz function (Fig. 5F). These results suggest that the C-terminal half of Garz contains signals that are essential for localization of the protein to the Golgi, and that the defects in garz embryos are due to the failure of the mutant proteins to localize correctly.

The ARF-GEF catalytic center is crucial for Golgi localization and function of Garz

The requirement of the C-terminal part of Garz for Golgi localization was somewhat surprising because the cycling of GBF1 between a cytoplasmic and a Golgi-membrane-associated pool was reported to depend on the nucleotide exchange reaction mediated by the Sec7 domain (Garcia-Mata et al., 2003; Niu et al., 2005; Zhao et al., 2006). To test whether more than one part of Garz is required for Golgi localization, and whether Garz localization also depends on ARF-GEF activity, we examined the localization of a mutant Garz protein, GFP–Garz(E740K). The E740K amino acid substitution in the catalytic center is presumed to abolish the nucleotide exchange reaction, as was shown for the corresponding mutations in the Sec7 domain of the human ARF-GEFs ARNO(E156K) (Beraud-Dufour et al., 1998) and GBF1(E794K) (Garcia-Mata et al., 2003). GFP–Garz(E740K) was localized predominantly at the Golgi in tracheal cells and in S2 cells, whereas cytoplasmic signals were strongly reduced compared with the distribution of wild-type GFP–Garz (Fig. 5I–L). Strikingly, tracheal expression of GFP–Garz(E740K) caused strong defects in apical secretion of Verm–RFP and in tracheal lumen dilation (Fig. 5J). The effect of GFP–Garz(E740K) on Verm–RFP secretion was reminiscent of the defects observed in garz loss-of-function mutants, but more severe in strength (Fig. 1E,F, Fig. 5J). These results suggest that GFP–Garz(E740K) interferes with the function of ARF G-proteins, possibly through a dominant-negative effect due to sequestration of ARF (Garcia-Mata et al., 2003; Szul et al., 2005) or titration of other Garz binding partners. Accordingly, GFP–Garz(E740K) on Verm–RFP secretion was reminiscent of the defects observed in garz loss-of-function mutants, but more severe in strength (Fig. 1E,F, Fig. 5J). These results suggest that GFP–Garz(E740K) interferes with the function of ARF G-proteins, possibly through a dominant-negative effect due to sequestration of ARF (Garcia-Mata et al., 2003; Szul et al., 2005) or titration of other Garz binding partners. Accordingly, GFP–Garz(E740K) is expected to interfere both with maternal and zygotic Garz protein pools, thus explaining the more severe phenotype than in zygotic garz mutants. We conclude that the activity and localization of Garz depend on the GDP-GTP exchange reaction mediated by the Sec7 domain, which is consistent with the notion that garz functions as an ARF-GEF. This conclusion is further supported by our finding that human GBF1 protein rescues the tube expansion and secretion defects of garz mutants (Fig. 3D,E). Similar GFP–Garz, HA-tagged GBF1 protein localizes to...
GM130-positive cis-Golgi structures in *Drosophila* S2 cells (supplementary material Fig. S3). Furthermore, as in mammalian cells, the E794K mutation leads to increased accumulation of GBF1 at the Golgi (supplementary material Fig. S3) (Garcia-Mata et al., 2003). These results suggest that ARF-GEF function and the mechanism of localization to the Golgi membrane are conserved between *Drosophila* Garz and human GBF1.

**garz is required for correct localization of COPI components and for Golgi morphology**

The human Garz homolog GBF1 plays a key role in COPI vesicle formation by recruiting and activating ARF1 at the cis-Golgi (Claude et al., 1999; Kawamoto et al., 2002; Zhao et al., 2002). To determine whether *garz* plays a similar role in *Drosophila*, we examined effects of *garz* mutations on the Golgi using markers for different Golgi compartments. A GFP fusion protein of the ARF1 homolog ARF79F (Shao et al., 2010) was enriched at Golgi stacks, whereas a fraction of the protein was uniformly distributed throughout the cytoplasm (Fig. 6A). In *garz* mutants, punctate localization of ARF79F–GFP was lost, and signals were instead distributed throughout the cytoplasm with few large GFP-positive dots (Fig. 6B), indicating that *garz* is required for ARF79F localization at the Golgi membrane. Consistent with this effect on ARF79F localization, *garz* function was also required for accumulation of the COPI coat subunit β′-COP in a punctate pattern in epidermal cells (Fig 6E,F) (Stempfle et al., 2010). Although β′-COP accumulation was rescued by expression of GFP–Garz in stripes of epidermal cells (Fig. 6F), finally, to address effects on the trans-Golgi network (TGN), we analyzed the distribution of the trans-Golgi marker GalT–GFP. Tracheal cells in wild-type stage 15 embryos contained multiple Golgi stacks labeled by GalT–GFP (Fig. 6C). By contrast, in *garz* mutants, GalT–GFP was diffuse and larger aggregates were observed, suggesting that the TGN becomes dispersed and Golgi aggregates are formed (Fig. 6D). Taken together, these results indicate that *garz* function is essential for the correct localization of the ARF1 homolog ARF79F and of COPI coatomer components, as well as for the normal organization of the Golgi complex.

**garz interacts genetically with the ARF1 homolog ARF79F and the ARF1-GAP homolog Gap69C**

Given the essential requirement of *garz* for ARF79F localization, we asked whether *garz* interacts genetically with the ARF G-protein machinery. To address this, we made use of a dominant phenotype caused by overexpression of Garz in the eye (Kraut et al., 2001; Raghu et al., 2009). Overexpression of Garz or GFP–Garz in the developing eye (using the GMR-Gal4 driver) causes a ‘rough’ eye phenotype, presumably because of overactivation of one or more ARF proteins (supplementary material Fig. S4). Consistent with this idea, co-overexpressing ARF79F–GFP with Garz or GFP–Garz enhanced the rough eye phenotype, whereas expression of ARF79F–GFP alone had no effect on eye morphology (supplementary material Fig. S4). Conversely, overexpression of Gap69C, a negative regulator of ARF79F (Raghu et al., 2009), suppressed the effect of *garz* overexpression in a similar manner to the suppression observed with *garz* RNAi as a control. By contrast, expression of a Gap69C RNAi construct enhanced the *garz* overexpression phenotype. We tested whether the *garz* overexpression effect could be suppressed by reducing
garz overexpression leads to ER retention and tube dilation defects

We also examined the effect of garz overexpression during embryonic tracheal development. Interestingly, although tracheal-specific expression of UAS-Garz or UAS–GFP–Garz rescued the tracheal lumen dilation and secretion defects of garz mutant embryos (Fig. 3C,E; data not shown), elevated expression (using two copies of btl-Gal4) of the same transgenes in a wild-type background led to defects reminiscent of the garz loss-of-function phenotype (Fig. 7C,E). Verm–RFP accumulated in tracheal cells and luminal diameter was significantly narrower than in wild-type embryos (Fig. 7G). Intracellular Verm–RFP localized in a perinuclear pattern (Fig. 7E) and colocalized with an ER marker (anti-KDEL; data not shown), suggesting that upon garz overexpression, Verm–RFP accumulates in the ER. This might be explained by an interaction of the overexpressed Garz protein with endogenous Garz or with other proteins in a dominant-negative fashion. However, both UAS–Garz and UAS–GFP–Garz were functional in a rescue assay (Fig. 3 and data not shown). Furthermore, garz overexpression led to enhanced punctate accumulation of ARF79F–GFP, whereas cytoplasmic ARF79F–GFP signals were reduced compared with levels in the wild type (Fig. 7B,F), suggesting that recruitment of ARF79F–GFP to the Golgi membrane was increased. By contrast, in garz mutants, the punctate localization of ARF79F–GFP was almost completely lost (Fig. 7D). These findings suggest that the secretion and tube dilation defects upon garz overexpression are not due to defective Golgi function or Golgi disassembly, but are rather due to increased ARF-GEF-dependent trafficking.

Discussion

We have described the function of the Sec7 domain protein Garz in epithelial tube development during Drosophila embryogenesis. We show that garz is essential for tracheal tube expansion and for epithelial protein secretion. Garz protein localizes to the Golgi and is required for normal Golgi morphology and for correct localization of COPI components. Garz is a member of the conserved Sec7-domain-containing family of large ARF-GEF proteins, which are involved in membrane-budding events at different intracellular compartments. Two families of large ARF-GEFs are known, the BIG and the GBF1 families. BIG1 and BIG2 localize to the TGN (BIG1 and BIG2) (Zhao et al., 2002) or recycling endosomes (BIG2) (Shin et al., 2004), where they facilitate recruitment of clathrin coat proteins. By contrast, GBF1 acts at the cis-Golgi membrane, where it initiates COPI vesicle formation by activating a class I ARF protein (Kawamoto et al., 2002; Garcia-Mata et al., 2003). Six different Sec7 domain proteins are encoded in the Drosophila genome. Although the functions of some of these ARF-GEFs have been studied using genetic approaches, mutations in garz, the single GBF1 family homolog in Drosophila, have not been described thus far. The garz gene was identified by virtue of its effects on axon guidance and eye development upon overexpression in the nervous system (Kraut et al., 2001; Shulman and Feany, 2003; Raghu et al., 2009). A recent study using tissue-specific RNAi showed that garz is required for normal development of the salivary glands (Szul et al., 2011). Here we describe the identification and characterization of garz loss-of-function mutations. We provide functional evidence that garz regulates tracheal tube expansion and secretion by acting as an ARF-GEF at the cis-Golgi. First, we show that Garz protein is distributed throughout the cytoplasm, whereas a fraction of the protein accumulates at the Golgi. Similarly, mammalian GBF1 protein dynamically cycles between cytoplasmic and Golgi membrane-associated pools (Niu et al., 2005; Zhao et al., 2006). Second, a catalytic site mutation that interferes with the nucleotide exchange reaction abolishes Garz...
function and leads to constitutive accumulation of Garz at the Golgi, suggesting that Garz function and subcellular distribution depend on GEF activity. Finally, we show that human GBF1 can substitute for garz function in Drosophila tracheal tube expansion and secretion, indicating that the relevant functions of Garz and GBF1 proteins have been evolutionarily conserved. Although GEF activity on ARF1 was shown to mediate the association of GBF1 with the Golgi membrane, it has been an open question how GBF1 protein initially gets recruited to the Golgi. The conserved regions outside the Sec7 domain are thought to play an important role in GBF1 localization. Our findings provide genetic evidence supporting an essential requirement of the C-terminal part of Garz protein for localization at the Golgi. The sequences missing in the truncated GFP–Garz(W982) version include the three conserved HDS motifs, suggesting that these motifs might be involved in recruitment of Garz to the Golgi. This is consistent with earlier work showing that a similar C-terminal truncation of human GBF1 protein also abolished the Golgi localization of GBF1 (Pan et al., 2008). Our finding that human GBF1 localizes to the Golgi in Drosophila cells and is able to rescue garz mutant phenotypes suggests that a conserved mechanism mediates localization of ARF-GEFs at the cis-Golgi in flies and mammals. Which interacting proteins are involved in localizing Garz to the Golgi? The HDS1 motifs of yeast Gea1 and Gea2 proteins were shown to physically interact through a conserved leucine residue with the Golgi membrane protein Gmh1 (Gea1-6 membrane-associated high-copy suppressor) (Chantalat et al., 2003). This leucine residue is conserved in human GBF1 (L987) and Drosophila Garz (L932), and is missing in the truncated Garz and GBF1 versions. However, although Gmh1 was shown to participate in the association of Gea proteins with membranes, it is not absolutely required for Gea2 localization at the Golgi (Chantalat et al., 2003). Thus, identification of other interacting proteins that mediate Golgi localization will be important to understand the function of Garz. A recent report implicated GBF1 and Garz in endocytic uptake of GPI-anchored proteins by a GPI-AP enriched early endosomal compartment (GEEC) (Gupta et al., 2009). Although we did not observe accumulations of GFP–Garz outside Golgi-positive spots in epithelial cells, the broadly distributed cytoplasmic signals might obscure localization of GFP–Garz at GEEC endosomes. Thus, our results do not exclude a dual role of Garz in COPI vesicle formation at the cis-Golgi and in GEEC endocytosis.

On which G-protein(s) does the Garz ARF-GEF act? Our results suggest that Garz acts on the Drosophila ARF1 homolog ARF79F, because accumulation of ARF79F–GFP at the Golgi requires garz function and garz interacts genetically with ARF79F and with Gap69C, a negative regulator of ARF79F. Consistent with this notion, overexpression of garz causes the redistribution of ARF79F–GFP from the cytoplasm towards a Golgi-localized pool. Similarly, overexpression of GBF1 in HeLa cells was reported to result in increased levels of ARF1–GTP (Honda et al., 2005). Taken together, our findings suggest that Garz regulates COPI vesicle formation as a rate-limiting factor for the recruitment and activation of the ARF1 homolog ARF79F, which is analogous to the roles of GBF1 and ARF1 in mammalian cells (Niu et al., 2005).

What is the role of garz in tracheal tube expansion? The tracheal tube dilation and secretion defects observed in garz mutants resemble the phenotype of mutants defective in COPI or COPII vesicle formation (Tsarouhas et al., 2007; Grieder et al., 2008; Jayaram et al., 2008; Förster et al., 2010; Norum et al., 2010). However, garz mutants exhibit weaker tracheal defects than γCOP mutants, and certain proteins, such as Pio, whose secretion is affected in γCOP mutants, appear unaffected in garz mutants (Grieder et al., 2008; Jayaram et al., 2008). This might be due to cargo-specific effects of the garz mutations, as was previously described for human GBF1 protein (Szul et al., 2007).

Fig. 7. Overexpression of garz causes tracheal lumen dilation and secretion defects resembling garz loss-of-function mutants. (A–F) Confocal sections of tracheal dorsal trunk of wild-type embryos (A,B), garz mutant embryos (C,D), and embryos overexpressing garz in tracheal cells (E,F). Whereas Verm–RFP is secreted into the tracheal lumen in wild-type embryos (A), Verm–RFP is retained intracellularly both in garz loss-of-function mutants (C) and in embryos overexpressing garz in tracheal cells under the control of two copies of btl-Gal4 (E). Note that garz mutants and garz overexpressing embryos show narrow tracheal lumina. (B,D,F) In wild-type embryos (B) ARF79F–GFP is localized in punctae, whereas a fraction of the protein is distributed in the cytoplasm. In garz mutants (D) punctate localization of ARF79F–GFP is abolished. Conversely, in garz overexpressing embryos (F) ARF79F–GFP accumulates exclusively in punctae, whereas cytoplasmic signals are reduced. Scale bars: 10 μm. (G) Quantification of tracheal lumen diameter in dorsal trunk metamere 6. Luminal diameter in garz mutant and garz overexpressing embryos is significantly narrower compared with the wild type (**P<0.001; error bars indicate s.d.).
Alternatively, perdurance of maternal garz gene products might partially mask the zygotic requirement of garz, resulting in an attenuated phenotype. Consistent with this idea, we showed that expression of dominant-negative garz<sup>Et408</sup>, which is expected to interfere with both maternal and zygotic Garz pools, causes more severe tracheal defects than those observed in zygotic garz mutants.

How does compromised COPI trafficking lead to defects in ER export? The retention of secreted proteins in the ER in garz mutants could be a secondary consequence of breakdown of the Golgi complex, resulting in reduced retrieval of ER-resident proteins. This might cause a block of ER export that is similar to that seen in COPII mutants (Tsrourhas et al., 2007; Förster et al., 2010; Norum et al., 2010). Alternatively, COPI vesicles might play a more direct role in the exit of secreted proteins from the Golgi. Interestingly, COPII-dependent exit of vesicles from the Golgi is required for expansion of the autophagosomal phagophore, a large membrane structure in yeast (van der Vaart et al., 2010), garz could be acting in a similar fashion by mediating the supply of Golgi-derived membrane material for apical plasma membrane growth during tracheal tube expansion. In both scenarios, compromised ARF-GEF-dependent trafficking leads to a reduction in the rate of apical membrane growth.

Our results suggest that the ARF-GEF Garz controls epithelial secretion and tracheal tube size by regulating membrane trafficking between the Golgi and the ER. To further the integrity and activity of the secretory apparatus, bidirectional membrane transport between these compartments has to be balanced. Under normal conditions GEFs are thought to be rate limiting for the initiation of vesicle formation. Thus, the effects of garz overexpression in tracheal cells and in the eye could be caused by hyperactivation of COPI retrograde trafficking. Similarly, overexpression of KDEL receptor was shown to cause the redistribution of Golgi-resident proteins to the ER (Hsu et al., 1992). By analogy, elevated retrograde trafficking in garz-overexpressing tracheal cells could reduce the rate of exocytosis through increased retrieval of proteins from the Golgi back to the ER, instead of passing through the Golgi. This could limit the supply of membrane material to the apical surface, resulting in narrow tracheal tubes. Although we cannot exclude the possibility that garz overexpression affects the Golgi in ways other than by triggering COPI vesicle trafficking, we did not observe obvious defects in Golgi morphology in garz-overexpressing cells. Furthermore, the garz overexpression phenotype in the eye is modulated by the dosage of a negative ARF regulator, Gap69c, suggesting that the overexpression effects are indeed due to elevated ARF activity. Thus, regulating the rate of COPI vesicle trafficking by changing the levels or activity of an ARF-GEF could provide a mechanism to control the rate of exocytosis. This might play an important role during epithelial morphogenesis, which involves dynamic membrane growth and remodeling.

Materials and Methods

**Drosophila strains**
The garz alleles W982, R1044, W1118, g31427 and g233 were isolated in an ethylmethane sulfonate mutagenesis screen. Mutations were induced on a balancer chromosome UAS-GFP UAS-Gal80-RFP chromosome ( Förster et al., 2010) and were balanced over the CyO Df(3R)Exel6061 (Parks et al., 2004), UAS-ARF79F-GFP (Shao et al., 2010), UAS-Gal4-GFP (Spaepen et al., 2004), UAS-Sec31-RFP ( Förster et al., 2010). UAS-Gap69c (Bagu et al., 2009), l(3)Gal4 (Shiga et al., 1996), UAS-lacZ<sub>EN</sub> (gift from Peter Gallant, Biocenter, University of Würzburg, Würzburg, Germany), UAS-Rab4-mRFP, 69B-Gal4 and GMR-Gal4 (Bloomington Stock Center). The following UAS-RNAi lines were obtained from the Vienna Drosophila Research Center (Dietzl et al., 2007): UAS-garz<sup>RNAi</sup> (VDRC 42140), UAS-Arf79F<sup>RNAi</sup> (VDRC 23082), UAS-Arf724<sup>RNAi</sup> (VDRC 17826), UAS-Arl100F<sup>RNAi</sup> (VDRC 12931), UAS-Arl4<sup>RNAi</sup> (VDRC 26007), UAS-Arl6<sup>RNAi</sup> (VDRC 41690), UAS-Arl9<sup>RNAi</sup> (VDRC 44358) and UAS-Gap69c<sup>RNAi</sup> (VDRC 26460, 107069).

**Tissue culture**

*Drosophila* S2R+ cells were cultured in Schneider’s medium (Invitrogen) and incubated at 27°C. For transfections, cells were seeded on coverslips in 24-well plates. Culture medium was replaced after 24 hours and cells were transfected with 1 μg plasmid DNA using FuGene HD transfection reagent (Invitrogen). 24 hours after transfection, cells were fixed with 4% formaldehyde and stained with antibodies and Hoechst 33342.

**Molecular biology**

Genomic DNA was prepared from homzygous garz embryos. Coding exons of the CGA487 gene, including exon–intron boundaries, were amplified by PCR. PCR products were purified and the entire garz coding region was sequenced on both strands (oligonucleotide sequences available on request).

Transgenic constructs were generated as follows. UAS-GFP-Garz: the EGFP coding sequence was inserted between the EcoRI and NotI sites of pUAST-attB (Bischof et al., 2007) to generate pUAST-attB-Egfp. The COPI<sub>β58</sub> coding region was amplified from the genomic BAC clone BCAR37109 (BACPAC Resources) using oligonucleotides KA16-NotI-F (ATAGCGGCCGCCATGGCGCGCCGTCACAGGCAC) and KA17-XbAl-R (ATATAGCTTCACTGCTTGGCCTAGAG). The PCR product was digested with NotI and XbaI and inserted into pUAST-attB-EGFP carrier gene. pUAST-attB-GFP could be generated using the GFP fragment from pUAST-attB-EGFP-Garz was excised by digestion with EcoRI and NotI. Overhangs were filled-in using Klenow DNA polymerase and the plasmid was re-ligated to generate pUAST-attB-garz. pUAST-attB-GFp-W982: the GFP-tagged N-terminal portion of the garz coding sequence corresponding to the garz<sup>W982</sup> allele was amplified from pUAST-attB-EGFP-Garz using oligonucleotides Ras42 (5′-CGAATTCATGGTGAGCAAGGGCGAG-3′) and Xba1W982 (5′-ATCATCTAGATCAGCTCTCTTAAAGCATTAC-3′), which contains the nonsense mutation of the garz<sup>W982</sup> allele (underlined). The PCR product was digested with EcoRI and XbaI and inserted into pUAST-attB-GFp-W982 using the oligonucleotides KA16-NotI-F (ATAGCGGCCGCCATGGCGCGCCGTCACAGGCAC) and KA50-NotI-R (TTATCAGCTCGGAGGGTCAG-3′) and inserted into pUAST-attB-GFp-W982. The EF740K point mutation was introduced by fusion PCR. Two PCR fragments were amplified using overlapping oligonucleotides containing the mutation (underlined; KA49, 5′-AAGGCTTACGATGTCATTTTTTG-3′ and KA50, 5′-AGATCAATGGACCCTTCGCCGCAATCCTGA-3′). The 5′ fragment of garz was amplified with KA16 and KA50, the 3′ fragment with KA49 and KA17. The resulting two fragments were joined by PCR using oligonucleotides KA16 and KA17. The PCR product was digested with NotI and XbaI and inserted into pUAST-attB-Egfp. pUAST-attB-HA-GFp1 and pUAST-attB-HA-GFp-E974K: wild-type and E974K mutant cDNAs of human GFp1 carrying an N-terminal HA tag (HA-GFp1 and HA-GFp-E974K) in the p5′TO vector were obtained from Paul Melancon (Department of Cell Biology, University of Alberta, Edmonton, Canada). The cDNAs were excised using Kpn1 and XbaI and inserted into pUAST-attB.

All PCR-amplified portions of the constructs were confirmed by DNA sequencing. pUAST-GFP-Garz was inserted into the attP landing site 86Fa on the third chromosome using F Carp4 integrase (Bischof et al., 2007). All other pUAST-attB transgenes were inserted into the attP landing site P2 at 68A4 on the third chromosome (Groth et al., 2004).

**RT-PCR**

Total RNA was extracted from 40 embryos (stage 16) per genotype using Trizol (Invitrogen). RNA was treated with DNaseI to remove residual genomic DNA. First-strand cDNA was generated using M-MuLV Reverse Transcriptase (Fermentas) and random hexamer and oligo-dT primers. Subsequent PCR (30 cycles) was performed using oligonucleotides that bind in exons 8 and 10 of the garz transcript. A fragment of the gfa2 (glutamine-fructose-6-phosphate aminotransferase 2, CG1345) transcript was co-amplified as an internal standard.

**In situ hybridization**

In situ hybridization was carried out according to standard procedures (Tautz and Pfeifle, 1989). To generate the garz antisense RNA probe, a 2 kb fragment from exons 7 and 8, including intron 7 (57 bp), was amplified from genomic DNA using oligonucleotides KA7-F (5′-TTACTAGCTCCTATGGAGCGCACGTCG3′) and KA15-T7-R (5′-TAACTAGCTCCTATGGAGCGCACGTCG3′). The reverse primer contained the T7 RNA polymerase promoter (underlined). Digoxigenin-labeled probes were generated by in vitro transcription with digoxigenin-labeled UTP (Roche) and detected using alkaline phosphatase-conjugated anti-digoxigen Fab fragments (1:2000; Roche).

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Garz regulates epithelial tube expansion

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References


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