mRNA Localization and ER-based Protein Sorting Mechanisms Dictate the Use of Transitional Endoplasmic Reticulum-Golgi Units Involved in Gurken Transport in *Drosophila* Oocytes

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The anteroposterior and dorsoventral axes of the future embryo are specified within *Drosophila* oocytes by localizing *gurken* mRNA, which targets the secreted Gurken transforming growth factor- α synthesis and transport to the same site. A key question is whether *gurken* mRNA is targeted to a specialized exocytic pathway to achieve the polar deposition of the protein. Here, we show, by (immuno)electron microscopy that the exocytic pathway in stage 9–10 *Drosophila* oocytes comprises a thousand evenly distributed transitional endoplasmic reticulum (tER)-Golgi units. Using *Drosophila* mutants, we show that it is the localization of *gurken* mRNA coupled to efficient sorting of Gurken out of the ER that determines which of the numerous equivalent tER-Golgi units are used for the protein transport and processing. The choice of tER-Golgi units by mRNA localization makes them independent of each other and represents a nonconventional way, by which the oocyte implements polarized deposition of transmembrane/secreted proteins. We propose that this pretranslational mechanism could be a general way for targeted secretion in polarized cells, such as neurons.

INTRODUCTION

Polarized localization of proteins is achieved by at least two different mechanisms. The first one relies on restricted localization of mRNAs that encode cytosolic proteins, allowing local protein translation, thus creating differential protein activity and generating cell asymmetry and polarity (Bashirullah *et al.*, 1998). This is, for instance, the case of *actin* mRNA localization in the nerve terminal of neurons (Lee and Hollenbeck, 2003), *oskar* localization at the posterior pole of *Drosophila* oocytes (Ephrussi *et al.*, 1991), and *ash1* localization in dividing yeast cells (Long *et al.*, 1997). On the other hand, the mechanism ensuring the polarized deposition of transmembrane or secreted proteins is thought, in mammalian cells, to be achieved by posttranslational sorting events in the *trans*-Golgi network (TGN). There, signals in their cytoplasmic tail or luminal domain are deciphered, resulting in the directed movement of specialized transport vesicles to allow specific deposition at the apical or basolateral plasma membrane of epithelial cells (Ikonen and Simons, 1998; Nelson and Yeaman, 2001).

However, there is a growing number of transmembrane and secreted proteins for which their transcript also exhibits a restricted localization. The transcript for the yeast plasma membrane protein Ist2 is actively transported along the acto-myosin network to the bud tip (Takizawa *et al.*, 2000), and the protein is deposited locally (Juschke *et al.*, 2004). *wingless* mRNA is localized apically in epithelial cells (Sim-

monds *et al.*, 2001). In a stage 9–10 *Drosophila* oocytes, *gurken* mRNA is transported and deposited exclusively in the dorsal/anterior corner (D/A; Neuman-Silberberg and Schupbach, 1993; Figure 1B). *gurken* encodes a protein that is synthesized in the endoplasmic reticulum (ER) as a 285-amino acid type I transmembrane protein precursor and transported to the Golgi apparatus where it is cleaved off by a specific protease, Brother of Rhomboid (Guichard *et al.*, 2000; Ghiglione *et al.*, 2002; Urban *et al.*, 2002), generating a luminal fragment belonging to the transforming growth factor- α family. This fragment is transported and released by exocytosis at the D/A corner in the intercellular space between the oocyte and the overlying follicle cells. There, it binds the epidermal growth factor receptor Torpedo in the plasma membrane of these follicle cells and induces a signaling cascade, so that they adopt their dorsal fate (van Eeden and St Johnston, 1999; Roth, 2003).

A key question regarding the restricted localization of transcripts encoding transmembrane/secreted proteins is how synthesis and transport is achieved, and whether they are sustained by a specialized exocytic pathway, near to where the transcripts are localized. We focus here on Gurken in *Drosophila* oocytes to address this issue.

In mammalian cells, the exocytic pathway comprises the continuous membrane bound organelle comprising a single lumen, the ER, where proteins destined to the extracellular medium and all membrane compartments of the cell (except mitochondria) are synthesized. Newly synthesized proteins exit the ER at numerous specialized ER exit sites, characterized by the presence of COPII-coated structures (also called transitional ER sites; tER sites) (Barlowe *et al.*, 1994; Bonifacino and Glick, 2004). The proteins reach the Golgi apparatus, a single copy organelle of striking morphology that comprises dozens of stacked Golgi cisternae, linked together

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Figure 1. Examination of the exocytic pathway in *Drosophila* oocytes. (A) Schematic representation of a stage 9–10 egg chamber with the oocyte abutting the 15 nurse cells and surrounded by a layer of somatic follicle cells. The dorsal/anterior corner is depicted in black (see *Materials and Methods*) and is where both Gurken protein and *gurken* RNA are known to localize. WT (B–D) and dCOG5-GFP (E and F) *Drosophila* egg chambers were processed for *gurken* RNA in situ hybridization (B), immunoelectron microscopy (C and E), conventional electron microscopy (D), and immunofluorescence (F). (C) Representation of a tER-Golgi unit (in brackets), according to the criteria described in the text, exhibiting a portion of an ER cisterna, one ER exit site, and a Golgi complex comprising a Golgi stack (G). (D) All tER-Golgi units visualized on the ultrathin resin stage 9 oocyte section are marked by an asterisk (*). (E) Ultrathin cryosections of dCOG5-GFP-expressing egg chambers were labeled for GFP (10-nm gold). The tER-Golgi unit (in brackets) is positive for dCOG5-GFP as were all the other in the oocyte cryosection. (F) Confocal section of a stage 9 dCOG5-GFP expressing egg chambers were labeled for GFP. The dots represent the tER-Golgi units in the oocyte, in the surrounding follicle cells and nurse cells. Note that the tER-Golgi units are randomly distributed within the ooplasm without concentration around the oocyte nucleus (N). The center of the oocyte shows a dimmer labeling intensity, due to penetration problems of the GFP antibody. Posterior, anterior, dorsal, and ventral are indicated by P, A, D, and V, respectively. N, nucleus. Bars, 200 nm (C and E); 5 μm (D and F).

by tubules to form a single large reticulum capping the nucleus (Mellman and Warren, 2000). It is the organelle where the newly synthesized proteins are posttranslationally modified and processed before reaching the TGN, where they are dispatched toward their correct final destination. The exocytic pathway is believed to function synchronously, that is, all the ER exit sites and all the stacks of the Golgi reticulum are involved in the transport of all proteins newly synthesized in the ER. Even in polarized cells, transmembrane proteins destined for the apical or basolateral plasma membrane are thought to be synthesized and transported together until they reach the TGN, where

they are sorted from one another to their appropriate location.

In *Drosophila*, the Golgi apparatus does not always exhibit a morphology of stacked cisternae. For instance, in imaginal discs of third instars, follicle cells of egg chambers, and syncytial embryos, it forms as a cluster of vesicles and tubules that are positive for several Golgi markers (Ripoche *et al.*, 1994; Kondylis *et al.*, 2001; Kondylis and Rabouille, 2003). Furthermore, the Golgi stacks when present are not linked together to form a single copy organelle. Instead, they are dispersed in the cytoplasm as it is in yeast (Rossanese *et al.*, 1999) and plants (Boevink *et al.*, 1998). Whatever the mor-

phology of the Golgi apparatus, they are in proximity to tER sites marked by the COPII subunit dSec23p. The resulting membrane structure (one tER site and one Golgi complex, found in the concavity of an ER cisterna; Figure 1C) is what we call a tER-Golgi unit, as it has been described in the yeast *Pichia pastoris* (Mogelsvang *et al.*, 2003). In *Drosophila* S2 cells, their number is definite (~20) (Kondylis and Rabouille, 2003).

We show here that the organization of the exocytic pathway in a stage 9–10 *Drosophila* oocyte (see King, 1970 for a description of the developmental stages) is the same as in *Drosophila* S2 cells, comprising multiple, seemingly identical tER-Golgi units (but now up to 1000) that are evenly distributed throughout the cell cytoplasm. We show that only a subset of them, situated at the D/A corner, are involved in the transport, processing and deposition of Gurken protein as described above. And we show, by using three *Drosophila* mutants in which *gurken* mRNA is mislocalized, that what controls the choice of the tER-Golgi units involved in Gurken transport, among the thousand present, is the localization of *gurken* mRNA. This pretranslational mechanism is coupled to posttranslational sorting events involving Cornichon and the transmembrane domain of Gurken that prevent its diffusion in the ER away from the D/A corner and ensure its efficient sorting to the tER-Golgi units at that same corner.

Together, these results suggest that tER-Golgi units can function independently from one another and that it is the mRNA localization coupled to sorting events in the ER that dictate their use and achieve polar distribution of transmembrane proteins.

MATERIALS AND METHODS

Fly Stocks

Oregon R and W¹¹¹⁸ are wild-type *Drosophila melanogaster*. Mutant fly stocks used are dCOG5-GFP (w;GFP-Fws/Cyo) (Farkas *et al.*, 2003); PDI-GFP (PDI::GFP/GFP-TM3,Sb) (Bobinnec *et al.*, 2003); *K10* (y[1] fs(1)K10[4] cv[1] v[1] f[1]/FM0) (Bloomington *Drosophila* Stock Center, Bloomington, IN); *squid¹* (Sqd¹/TM3, Sb Ser) (Norvell *et al.*, 1999); *cnj^{AR55}* (b *cnj^{AR55}* pr *cn*/Cyo), *cnj^{CF5}* (b *cnj^{CF5}* pr *cn* wx bw/Cyo) and *cnj^{AA12}* (b *cnj^{AA12}*/Cyo) (Roth *et al.*, 1995); *grk^{2B6}* (*grk*[2B6]b *cn* s L bo/Cyo) and *grk^{2E12}* (w; *grk*[2E12]b/Cyo) (Neuman-Silberberg and Schupbach, 1993); and fly lines expressing Gurken lacking the transmembrane and the cytoplasmic domain gATC4.1 and the cytoplasmic domain gΔC100 (Queenan *et al.*, 1999). The stocks carrying the constructs encoding the truncated versions of Gurken proteins were crossed into the *grk^{2B6}* background and females from this cross were mated with male *grk^{2E12}* to yield orange-eyed black flies (gATC4.1; *grk^{2B6}*/*grk^{2E12}* and gΔC100; *grk^{2B6}*/*grk^{2E12}*). All are raised on normal cornmeal-agar medium at 25°C. The *merlin* thermosensitive mutant *yw ts594* (*mer^{ts1}*; MacDougall *et al.*, 2001) was induced by shifting virgin flies to 29°C for 3 d. Ovaries were prepared by dissecting 2- to 3-d-old females (fed with extra yeast) in *Drosophila* Ringer buffer. They were transferred to the appropriate fixatives as whole ovaries.

Antibodies

The antibodies used are the monoclonal mouse anti Gurken antibody 1D12 (Developmental Studies Hybridoma Bank, Iowa City, IA; Queenan *et al.*, 1999); the anti-mammalian Sec23p rabbit polyclonal antibody (Affinity BioReagents, Golden, CO; Kondylis and Rabouille, 2003); the crude anti Yolkless rabbit polyclonal antibody 155 (gift from A. Mahowald, Chicago, IL); and the polyclonal anti-green fluorescent protein (GFP) antibody A6455 (Molecular Probes, Leiden, The Netherlands).

Electron Microscopy

Ovaries were dissected and fixed with trialddehyde (5% glutaraldehyde [GA], 2% paraformaldehyde [PFA], 1% acrolein, 2.5% DMSO in 0.08 M cacodylate buffer, pH 7.4) for 3 h at room temperature. This was followed by postfixation and embedding in Spurr's low viscosity resin. Ultrathin resin sections were cut on a Reichert Ultra Microtome. Ovaries also were fixed in 4% PFA in 0.1 M phosphate buffer (PB), pH 7.4, for 3 h at room temperature (RT) followed by overnight at 4°C, or in 2% PFA and 0.2% GA in the same buffer for 3 h at RT, followed by overnight at 4°C, and stored in 1% PFA in PB at 4°C. The individual egg chambers were embedded in 12% gelatin (Liou *et al.*, 1996) and

mounted on aluminum pins and frozen in liquid nitrogen so that they could be cut along the long axis on a Reichert Ultracut S cryotome at −120°C.

Immunoelectron Microscopy (IEM)

Sixty-nanometer-thick cryosections were incubated with antibodies described above. Rabbit antibodies were recognized by protein A conjugated to 10- or 15-nm gold particles. Mouse antibodies were detected by a rabbit anti-mouse IgG (DakoCytomation Denmark A/S, Glostrup, Denmark) followed by either one of the protein A gold complexes. Double labeling procedures were performed sequentially as described in Slot *et al.* (1991).

In Situ Hybridization

In situ hybridization on whole mount ovaries (fixed for 15 min in 4% PFA in phosphate-buffered saline [PBS]) was carried out according to Tautz and Pfeifle (1989) with minor modifications. Digoxigenin-labeled *gurken* RNA-probe was produced from the 1.7-kb *gurken* cDNA (Neuman-Silberberg and Schupbach, 1993) by using a DIG RNA labeling kit according to manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany).

Immunofluorescence

Ovaries were dissected and fixed in 4% PFA in PBS for 15 min at RT. After three washes in 0.3% Triton X-100 in PBS (PBT), ovaries were first incubated in 1% Triton X-100 in PBS for 30 min, followed by 1-h block in PBT with 2% bovine serum albumin (BSA) followed by the incubation with the primary antibodies in PBT supplemented by 0.5% BSA for 3 h at RT or O/N at 4°C. Ovaries were washed in PBT three times in 1 h before incubation with the secondary IgG coupled to Alexa 488 for rabbit antibodies, or Alexa 568 for mouse antibodies (1:500; Molecular Probes) in 0.5% BSA in PBT for 2 h at RT. Ovaries were washed in PBT, mounted in Vectashield, and viewed under a Leica TCS-NT confocal microscope.

Fluorescence Recovery after Photobleaching (FRAP) Analysis

Protein disulfide isomerase (PDI)-GFP transgenic females were directly dissected in halocarbon oil series 95 on a glass slide. Ovarioles were separated and covered with a coverslip. The GFP-signal was analyzed directly by using a Leica TCS-NT confocal microscope with a 40× objective and scanned at 2× zoom at 25% laser power (prebleach). The photobleaching was performed by scanning a distinct area at 32× zoom, (square box in Figure 7, A–D) at full laser power for 30 s. Recovery of the GFP signal was analyzed at 2× zoom with 25% laser power, every 30 s.

Drug Treatments

WT or *cnj^{AR55}/cnj^{CF5}* ovaries were directly dissected in serum enriched Schneider insect medium supplemented with either 0.4% ethanol (control) or 90 μM brefeldin A (BFA; dissolved in 80% ethanol; Sigma-Aldrich, St. Louis, MO). The ovaries were incubated for 15 min at 37°C, followed by 90 min at 25°C (as described for *Drosophila* S2 cells, Kondylis and Rabouille, 2003), followed by immunofluorescence processing.

Quantitation

tER-Golgi units are described in the text and in Kondylis and Rabouille (2003). For estimating the total number of the tER-Golgi units in a stage 9 oocyte, the resin embedded oocytes were serial sectioned. Only one 50-nm section every 500 nm of tissue was picked up. This is because a tER-Golgi unit is comprised within a sphere of 500 nm in diameter, so the tER-Golgi units in each section selected should only be counted once. We counted the total number of tER-Golgi units through the stacks of selected sections for two oocytes and estimated both their total number and their density. The density was verified on any other single section through at least 20 different oocytes.

The labeling density of Gurken within the tER-Golgi units was estimated by point hit as described previously (Kondylis and Rabouille, 2003). We also measured the distance between the most D/A plasma membrane point and the Gurken-positive tER-Golgi units. Pictures of the dorsal anterior corner of wild-type (WT) oocyte cryosections were taken at 6000×, printed, and assembled to visualize both the Gurken-labeled tER-Golgi units and the nonlabeled ones. Because *gurken* mRNA is localized between the nucleus and the plasma membrane at the D/A corner, we took the point representing this corner as the reference point and measured the distance between this point and the labeled tER-Golgi units. We verified the distance on immunofluorescence pictures where individual tER-Golgi units are visualized. We used a similar method for *merlin* mutants where the most posterior point of the oocyte was the point of reference.

We also measured the area of cytoplasm where *gurken* mRNA is localized and where most of the labeled tER-Golgi units are situated. This area is described as a triangle where the point representing the D/A corner is the top angle opposite to the line crossing the nucleus in the middle minus the nucleus itself (Figure 1A, in black). The area of cytoplasm was estimated by point hit.

RESULTS

The Exocytic Pathway in the Drosophila Oocyte Comprises Multiple tER-Golgi Units That Are Uniformly Distributed throughout the Ooplasm

To determine whether the exocytic pathway was restricted to a particular region of the ooplasm (for instance at the D/A corner), or present all over, we investigated the organization of this pathway in stage 9 oocytes by electron and immunofluorescence microscopy. At that stage, the oocyte is a cell with an anterior/posterior length of 30–40 μm and a dorsoventral diameter of 50–70 μm . We found that the oocyte, like other *Drosophila* cells, comprises multiple tER-Golgi units, some exhibiting a Golgi stack organization (Figures 1C and 2A), and others Golgi clusters (Figure 2C). They are comprised within a sphere of $\sim 400 \pm 100$ nm in diameter and are positive for the Golgi tethering factor COG5 (Figure 1E), a subunit of the COG complex, tethering ER-derived vesicles to Golgi membrane (Whyte and Munro, 2001), that is also known in *Drosophila* as Four Way Stop or dCOG5 (Farkas *et al.*, 2003), and positive for dSec23p (Figure 2), a COPII subunit marking the tER sites (Kondylis and Rabouille, 2003).

We serial sectioned stage 9 oocytes along the long axis and estimated that they contain 1000 ± 200 tER-Golgi units in total. They were randomly distributed throughout the ooplasm (Figure 1D, asterisks) with the same density near and around the nucleus, a landmark for the D/A corner (1unit/ 26.6 ± 5.1 μm^2 section), at the ventral, anterior, and posterior sides (1unit/ 22.2 ± 4.1 μm^2 section). This random distribution was confirmed by examining confocal projections of stage 9 oocytes expressing dCOG5-GFP (that is present in all tER-Golgi units of the oocyte; Figure 1E) either live, or fixed and postlabeled with an anti GFP antibody (Figure 1F).

Gurken Protein Is Only Transported through the tER-Golgi Units at the D/A Corner

Gurken protein exhibits a restricted and polar localization at the D/A corner of a stage 9–10 oocyte (Neuman-Silberberg *et al.*, 1996). The tER-Golgi units that make up the oocyte exocytic pathway are evenly distributed. Therefore, we predict that Gurken is transported and processed exclusively in the tER-Golgi units present at the D/A corner.

To test this hypothesis, we cryosectioned fixed stage 9 oocytes and visualized Gurken by IEM. As expected, a fraction of gold particles corresponding to Gurken (ranging from 10 to 20%) was found in the space between the oocyte and the overlying follicle cells of the D/A corner. However, the bulk of Gurken was found within the oocyte at the D/A corner between the nucleus and plasma membrane. About 20% of intracellular Gurken was present in the local endoplasmic reticulum, consistent with its site of synthesis. The remainder was found in 10 ± 2 tER-Golgi units/section (Figure 2A), 95% of them situated in the region 1 of the cytoplasm encompassed within the triangle as shown in Figures 1A and 2A (see *Materials and Methods* for definition). This region has an area of 225 ± 50 μm^2 and is where *gurken* mRNA is known to localize (Figure 1B). These Gurken-positive tER-Golgi units (comprising either a Golgi stack or a Golgi cluster) exhibited a labeling density for Gurken of 26 ± 12 gold/ μm^2 and were situated not further than 20 μm from the most D/A corner (see *Materials and Methods* for definition) with an average distance of 12.2 ± 3.0 μm . We also found 5% of Gurken-positive units at longer distance (up to 30 μm), but their labeling density was much weaker (3 ± 2 gold/ μm^2).

Figure 2. Gurken in the tER-Golgi units at the D/A corner. WT egg chamber cryosections were double labeled for Gurken (10-nm gold) and dSec23p (15-nm gold). Different areas of the oocyte sections were examined. (A) Area 1 corresponds to the D/A corner (see *Materials and Methods*, inset; the nucleus serves as a landmark). Gurken protein (arrows) is present in all the tER-Golgi units in this area that are also labeled for dSec23p. (B) Area 2 corresponds to the anterior side of the oocyte (inset) toward the middle. (C) Area 3 corresponds to the V/A corner (inset). In areas 2 and 3, the tER-Golgi units are negative for Gurken protein but positive for dSec23p. Bar, 200 nm (inset, 5 μm).

Away from the nucleus, at a distance higher than 30 μm along the anterior or dorsal side, Gurken was mostly absent from the extracellular space, and the tER-Golgi units comprised within the considered areas were negative for Gurken

Figure 3. Gurken colocalizes with dCOG5-GFP at the D/A corner. (A) Stage 9–10 WT egg chambers were processed for immunofluorescence and labeled for Gurken (red). Gurken is present in dots around the oocyte nucleus (N), in the ER at the D/A corner (white arrows), and in the intercellular space between the oocyte and the follicle cells at the D/A corner (white arrowheads). (B–D) dCOG5-GFP-expressing egg chambers were double labeled for Gurken (B, red) and GFP (C, green). Note that the Gurken-positive dots show complete overlap with dCOG5-GFP (D, merge) and therefore represent individual tER-Golgi units. Note that the Gurken in the ER (white arrows) does not overlap with dCOG5-GFP. The number of Gurken-positive tER-Golgi units is higher than 10 (see *Results*), because the thickness of the confocal section presented here is 3 times higher than this of cryosections. The egg chambers are oriented as in Figure 1A. Bar, 5 μ m (A and D).

(our unpublished data). So were the tER-Golgi units in the middle of the oocyte (Figure 2B, area 2), at the posterior pole (our unpublished data) and at the ventral/anterior corner (Figure 2C, area 3). However, all units were positive for dSec23p (Figure 2).

We also visualized Gurken by immunofluorescence in WT oocytes and were able to distinguish the tER-Golgi units involved in its transport (Figure 3A). These units were represented by dots around the nucleus, at the D/A corner and were positive for dCOG5-GFP (Figure 3, B–D), and dSec23p (our unpublished data). As expected, Gurken also was observed at the plasma membrane (Figure 3, arrowheads) and in the ER at the D/A corner (arrows) where it does not colocalize with dCOG5-GFP. These results confirmed that only tER-Golgi units in the area of cytoplasm around the nucleus, at the distance not $>20 \mu$ m from the outmost D/A corner, were involved in Gurken transport and processing.

This result shows that within a single cell, proteins can be transported through a specific subset of tER-Golgi units,

suggesting that the functioning of these units can be independent of each other.

All tER-Golgi Units Are Functional and Transport Yolkless

One way to explain this result, though, is to argue that the Gurken-positive tER-Golgi units are the only functional ones in the oocyte. We therefore tested the transport of Yolkless, a transmembrane receptor present over the entire oocyte plasma membrane, and in the oocyte endosomal compartment, where it acts as the receptor for vitellogenin proteins synthesized by the follicle cells (Schonbaum *et al.*, 1995, 2000). These vitellogenin proteins, once endocytosed, are stored in yolk granules, on which the egg feeds once it is fertilized.

Yolkless is synthesized and transported to the plasma membrane through the oocyte exocytic pathway. In stage 9–10 egg chambers, Yolkless could be observed in transit in all tER-Golgi units observed in all areas of the oocyte, for instance, at the ventral side (Figure 4B), at the posterior pole (Figure 4C), and at the D/A corner together with Gurken (Figure 4A). This shows that all tER-Golgi units present in the ooplasm are functional. It also reinforces the statement of independent functioning of the tER-Golgi units.

It Is Gurken mRNA Localization That Dictates the Choice of tER-Golgi Units Used for Gurken Transport

If, within an oocyte, functional tER-Golgi units are evenly distributed, how is the selection of those involved in Gurken transport achieved and implemented? Because *gurken* mRNA is localized at the D/A corner, we hypothesize that the choice of these tER-Golgi units is dictated by the restricted localization of *gurken* mRNA. If this is true, ectopically or mislocalized *gurken* mRNA should recruit other tER-Golgi units away from the D/A corner.

To test this hypothesis, we first used *merlin* mutants in which *gurken* mRNA remains localized at the posterior pole till later stages (including 9 and 10; MacDougall *et al.*, 2001). In WT, at the end of stage 7, Gurken signaling at the posterior pole follicle cells is answered by their sending a signal back to the oocyte, which leads to the change of microtubule polarity. As a consequence, the nucleus moves from the posterior pole toward the dorsal anterior corner, and *gurken* mRNA also is found localized at this corner (van Eeden and St Johnston, 1999; Roth, 2003). In *merlin* mutants, the posterior follicle cells do not send the signal back to the oocyte, the microtubules do not change their polarity, with the result that, in 55% of the cases, the nucleus and *gurken* mRNA remain at the posterior pole (MacDougall *et al.*, 2001; Figure 5A).

We localized Gurken protein in stage 9–10 *merlin* oocytes by immunofluorescence and IEM. Gurken was found in the space between the oocyte and the posterior follicle cells (our unpublished data) as well as in endosomes in follicle cells (Figure 5B, arrows), in agreement with its signaling. Furthermore, only the tER-Golgi units located at the posterior pole were positive for Gurken (Figure 5, B and C). They were located not further away than 20μ m from the most posterior point, where the mRNA is located. Their labeling density for Gurken was 27.1 ± 11.0 gold/ μ m², in agreement with the WT situation at the D/A corner. The anterior tER-Golgi units were totally negative (Figure 5D). This suggests that mRNA localization can indeed dictate the usage of tER-Golgi units located next to it, in the D/A corner for a stage 9–10 WT oocyte, and at the posterior pole for an equivalent *merlin* oocyte.

Figure 4. Yolkless is transported in all tER-Golgi units. Cryosections of stage 9–10 WT egg chambers were labeled for the Gurken (15 nm) and Yolkless (10 nm). Yolkless, on its way to the plasma membrane, passes through all the tER-Golgi units of the ooplasm, at the D/A corner (A, together with Gurken), at the V/A corner (B) and at the posterior pole (P) (C). Arrows mark the specific Yolkless labeling. The 10-nm gold in A that is not marked by arrows is an artifact due, in this instance, to the double labeling technique. Bar, 200 nm.

Figure 5. Gurken protein in the posterior tER-Golgi units of *merlin* mutants. Homozygous stage 9–10 *merlin* egg chambers were processed for *gurken* mRNA in situ hybridization (A), immunofluorescence of Gurken protein (B), and cryosectioned and labeled for Gurken (15-nm gold) (C and D). Note that only the tER-Golgi units next to the oocyte nucleus (N) at the posterior pole are Gurken positive (C), whereas these of the anterior side are negative (D). The white arrows in B point to Gurken-positive structures in posterior follicle cells, representing presumably endosomes, and the white arrowheads point to tER-Golgi units at the oocyte posterior pole. The dashed white line represents the contours of the oocyte. The egg chambers are oriented as in Figure 1A. P, posterior pole. Bar, 200 nm.

To test this prediction further, we used the *fs(1)K10* and *squid¹* mutants, where *gurken* mRNA is no longer restricted to the D/A corner, but delocalized along the anterior cortex and at the ventral/anterior corner (Serano *et al.*, 1995; Norvell *et al.*, 1999; Figure 6, A and E). *gurken* mRNA localizes to an asymmetric ring along the anterior side of the oocyte with a weaker intensity toward the ventral side. The translation and deposition of Gurken protein is nevertheless sustained (Serano *et al.*, 1995; Norvell *et al.*, 1999; Figure 6). As ex-

Figure 6. Gurken protein in the tER-Golgi units of *K10* and *squid¹* mutants. Homozygous *K10* (A–D) and *squid¹* (E–H) egg chambers were processed for *gurken* mRNA in situ hybridization (A and E), or cryosectioned and labeled for Gurken (15-nm gold) (B–D, F–H). Different areas of the oocyte sections were examined. Area 1 (B and F) corresponds to the D/A corner (the oocyte nucleus [N] serves as a landmark), area 2 (C and G) corresponds to the anterior side of the oocyte, and area 3 (D and H) corresponds to the V/A corner (see legend for Figure 2). The tER-Golgi units (in brackets) were all positive for Gurken protein. Bar, 200 nm.

pected, Gurken labeling was present in the tER-Golgi units at the D/A corner (Figure 6, B and F) with a labeling density of 23.5 ± 11.0 gold/ μm^2 for *K10* mutants, and 21.5 ± 10.0 gold/ μm^2 for *squid¹*, comparable with WT (Figure 2). But in both mutants, Gurken also was present in the tER-Golgi units along the anterior side (Figure 6, C and G, with a Gurken labeling density of 8.5 ± 5.0 for *K10*, and 6.5 ± 2.5 for *squid¹*), and at the ventral/anterior corner (Figure 6, D and H, with a Gurken-labeling density of 8.5 ± 6.0 for *K10*, and 5.5 ± 3.5 for *squid¹*), in a sharp contrast to WT (Figure 2, B and C). The slightly decreased Gurken-labeling density along the anterior axis and at the ventral/anterior corner is in agreement with the weaker RNA labeling observed (Figure 6, A and E). The space between the oocyte and the nurse cells was also weakly positive for Gurken (our unpublished data).

That mislocalized *gurken* mRNA was able to induce the local synthesis, transport, and processing of full-length Gurken protein was suggested by Serano *et al.* (1995) and Norvell *et al.* (1999). Our results show here that this is sustained by polarized exocytosis through selected tER-Golgi units (and post-Golgi compartments) situated in proximity to, and chosen by, *gurken* mRNA. This suggests,

then, that mRNA localization dictates the use of the machinery for processing and transport of transmembrane/secreted proteins.

A Very Active Transport Out of the ER

Additional mechanisms might ensure that local synthesis of Gurken is followed by local polarized delivery at the D/A intercellular space. To achieve this, diffusion of newly synthesized Gurken must be restricted, either because the ER is discontinuous and Gurken cannot go beyond the stretch of ER in which it has been synthesized (for instance, around the nucleus at the D/A corner), or because Gurken is transported very efficiently out of the ER as soon as it is synthesized.

In eukaryotic cells, the ER is continuous throughout the entire cytoplasm. In *Drosophila* oocytes, the visualization of the ER-resident protein protein disulfide isomerase tagged with GFP (PDI-GFP) shows that the ER pervades the entire oocyte (Bobinnec *et al.*, 2003).

We show here that the ER does not only pervade the oocyte but also comprises a single lumen. We first performed a FRAP (Nehls *et al.*, 2000) experiment on the PDI-GFP-expressing egg chambers (Figure 7, A–D). Because

Figure 7. The ER is continuous in the *Drosophila* oocyte. (A–D) FRAP experiment on PDI-GFP egg chambers shows that the ER pervades the oocyte and comprises a single lumen. Photobleaching was performed for 30 s at 100% laser power in a selected area (square box, B), but diffusion from the surrounding ER enables recovery of the GFP signal (C), which is fully restored after 5 min (D). Cornichon mutants (strong allelic combination *cni^{AR55/cni^{AA12}}*) were labeled for Gurken protein by immunofluorescence (E and F) or IEM (G–J; 10-nm gold, arrows) and for mRNA (K and L). In this genetic background, the nucleus fails to move to the D/A corner in 70% of the oocytes and is localized at the posterior pole (F and L). The IEM micrographs (G–J) were generated from such an oocyte, and Gurken is present throughout the ER in all areas, reaching the anterior side after being synthesized at the posterior pole (L). The tER-Golgi units were not labeled. The egg chambers are oriented as in Figure 1A. N, oocyte nucleus. Bars, 10 μm (E and F); 100 nm (G–J).

PDI-GFP is resident in the ER, it can be used as a marker for diffusion in this organelle. By bleaching a certain area in the oocyte (boxed area in Figure 7A), the GFP signal is initially lost (Figure 7B) but recovers (Figure 7C) and is restored completely (Figure 7D) in ~5 min. This result can only be explained by flow of not-bleached PDI-GFP into the bleached area, from the surrounding ER, showing the continuity. A similar result was obtained when bleaching any region of the oocyte ER, including the D/A corner.

We confirmed that the ER is continuous by using *cornichon* (*cni*) mutants. Cornichon is a protein of 144 amino acids, is predicted to contain three transmembrane domains, has been described as the potential Gurken receptor at the tER sites (Roth *et al.*, 1995), and is the putative orthologue of Erv14p (Powers and Barlowe, 1998). In the strong allelic combination of *cni^{AR55/cni^{AA12}}* mutants (leading to truncated proteins), Gurken is retained intracellularly (Roth *et al.*, 1995) and is observed throughout the entire oocyte (Figure 7, E and F). Although it is still concentrated near the nucleus at the D/A corner (Figure 7E) or at the posterior pole (Figure 7F) when oocyte development has been impaired due to the mutation (Roth *et al.*, 1995), we show here that it exclusively labels the ER throughout the oocyte (Figure 7, G–J) and that the tER-Golgi units are not labeled, suggesting that Gurken sorting has not been achieved.

This ER-retained Gurken protein was produced from a single source of mRNA localized near the nucleus, at the D/A corner (Figure 7K), or at the posterior pole (Figure 7L). This shows that the ER can be filled up entirely by from a single localized source of translation, thus indicating that it is connected throughout the oocyte, including the D/A corner.

In this continuous ER, diffusion of newly synthesized Gurken protein within the plane of the organelle membrane is in principle expected before it is further transported toward the tER-Golgi units. However, Gurken does not seem to diffuse and is possibly transported very efficiently out of the ER. We set out to investigate several mechanisms responsible for this efficient sorting.

First, as described above, Cornichon provides the first of such a mechanism; when its function is impaired, Gurken is present in the ER and not transported to the tER-Golgi units (Figure 7, E–J).

Second, different domains of Gurken protein provide information for its efficient sorting. Gurken transmembrane domain has been suggested to be necessary for the efficient transport of the protein. The truncated Gurken protein lacking its transmembrane and cytoplasmic domain (gΔTC) has been shown to be retained intracellularly (Queenan *et al.*, 1999). When gΔTC is expressed in a Gurken protein null background (*grk^{2E12/grk^{2B6}}*), the truncated protein is present throughout the entire ER as seen by immunofluorescence (Figure 8, A and B) and by IEM (our unpublished data). As a result, it cannot signal to the adjacent follicle cells and rescue the *gurken* mutant phenotype. This result suggests that gΔTC is locally synthesized and diffuses throughout the oocyte ER, suggesting that the truncated domains are crucial for its efficient sorting at the D/A corner. A truncated version of Gurken lacking only its cytoplasmic domain (gΔC) does not show such a strong diffusion and fully rescues the *grk^{2E12/grk^{2B6}}* phenotype (Queenan *et al.*, 1999). Its diffusion in the ER was nevertheless slightly broader than in the WT (our unpublished data). This shows that it is the transmembrane domain of Gurken that is mostly necessary for efficient exit from the ER.

We further investigated whether the diffusion of WT Gurken in the ER could be induced by chemically blocking its exit from the ER by using the drug BFA. Under BFA treatment, transmembrane proteins that normally would be transported to the Golgi apparatus are trapped and diffuse in the ER (Lippincott-Schwarz *et al.*, 1989; Altan-Bonnet *et al.*, 2004). Treatment of WT oocytes with BFA had a moderate effect of retention and diffusion of Gurken in the ER and was observed only in ~30% of the egg chambers, even when incubations with the drug were extended to 3 h (Figure 8D). In 70% of the treated egg chambers, the pattern did not differ

Figure 8. Gurken diffusion in the ER can be induced. Gurken protein null background (*grk^{2B6}/grk^{2E12}*) egg chambers expressing the truncated form of Gurken lacking its transmembrane and cytoplasmic domain (*gΔTC*) (A and B), WT egg chambers (C and D), and *cni^{CF5}/cni^{AR55}* (E and F), mock- (C and E) and brefeldin A-treated (D and F) were labeled for Gurken for immunofluorescence. Note that Gurken has diffused in the ER when it is truncated and when the egg chambers are treated with BFA (arrows in D and F). The egg chambers are oriented as in Figure 1A. N, oocyte nucleus. Bar, 10 μ m.

from the mock-treated ones (Figure 8C), and Gurken was present in tER-Golgi units and the ER in the D/A corner. We reasoned that perhaps, in WT oocytes, Cornichon was binding Gurken and trapped it at the tER sites, possibly in COPII-coated vesicles, even though the movement of cargo to the Golgi apparatus is inhibited by the BFA treatment (Altan-Bonnet *et al.*, 2004).

We tested this notion by performing the BFA treatment in a weak allelic combination of *cornichon*, *cni^{AR55}/cni^{CF5}*. The *CF5* allele produces a Cornichon protein that is 22 amino acids larger than the WT because of a mutation in the stop codon (Roth *et al.*, 1995). In the absence of the drug, the intracellular Gurken pattern in this mutant is almost indistinguishable from the WT (compare Figure 8, C and E);

Gurken is found in tER-Golgi units with a fraction also observed in the ER, but its secretion is moderately impaired, leading to a weak signaling to the D/A follicle cells and ventralization of the oocytes (our unpublished observation). In the presence of BFA, though, Gurken diffuses further in the ER in 75% of the observed egg chambers. This indicates the importance of Cornichon as a protein required for capturing newly synthesized Gurken in the ER and directing it to the most proximal tER-Golgi units. This also suggests, as in mammalian cells, that BFA can be efficient at affecting the ER to Golgi dynamics and retrieving Golgi localized proteins (such as Gurken) in the ER.

Together, this suggests that the pretranslational mechanism (through *gurken* mRNA localization) that implements the polar synthesis of Gurken at the D/A corner is followed by strong sorting events of the protein from the ER, preventing its diffusion and ensuring its delivery at the D/A intercellular space where it can perform its biological function.

DISCUSSION

The tER-Golgi Units at the D/A Corner Are Seemingly Identical to the Others

To understand how Gurken, as a transmembrane protein, achieves its polar distribution, we elucidated the organization of the exocytic pathway in *Drosophila* oocytes. We found that it is similar to other *Drosophila* cells observed so far. Namely, it contains a continuous ER that pervades the entire ooplasm (Bobinnec *et al.*, 2003; this study), from which a multitude of tER-Golgi units arise. In the oocyte, like in S2 cells, the tER-Golgi units comprise an ER exit site (positive for dSec23p), closely apposed to a Golgi apparatus, either under the form of a cluster of vesicles and tubules, or a Golgi stack, both marked by the Golgi marker, dCOG5. S2 cells contain ~20 of these units, whereas the number is much greater in oocytes (~1000; this study) but with an equivalent density (S2 cells have about a 60–100 times smaller volume than a stage 9 oocyte).

One way to explain Gurken deposition at the D/A corner is to argue for a concentration of the tER-Golgi units at this corner. Recently, it has been shown (Preisinger *et al.*, 2004) that cell migration in wound healing was accompanied by the redistribution and concentration of their Golgi complex to the part of the cell facing the injury, thus sustaining a polarized secretion that helps in the healing. We show, using immunofluorescence and electron microscopy, that the thousand tER-Golgi units in the *Drosophila* oocyte are evenly distributed throughout the ooplasm. This concentration is therefore unlikely to be the underlying mechanism for Gurken polarity.

Another way to explain that Gurken is only synthesized and transported in the tER-Golgi units localized at the D/A corner is to argue that they have a unique composition in regard to the known three proteins involved in its movement through the exocytic pathway: Star (Pickup and Banerjee, 1999), Cornichon (Roth *et al.*, 1995), and Brother of Rhomboid (Brho; Guichard *et al.*, 2002). Perhaps these three proteins only reside in the tER-Golgi units at the D/A corner, therefore rendering them, and only them, competent for Gurken transport. Star is thought to act as an ER chaperone helping in the exit from the ER (Ghiglione *et al.*, 2002; Urban *et al.*, 2002). Cornichon is a potential Gurken receptor at the ER exit sites (Roth *et al.*, 1995). Brho is a specific endoprotease located in the Golgi apparatus (Ghiglione *et al.*, 2002; Urban *et al.*, 2002) that cleaves Gurken just after its transmembrane domain, thus generating the luminal active li-

gand of Torpedo, and a C-terminal membrane bound fragment whose fate is undetermined.

Several lines of evidence, however, suggest that these proteins are not restricted to the tER-Golgi units of the D/A corner. First, *cornichon* and *brho* mRNAs do not have a polarized localization, but rather occupy the entire oocyte (Roth *et al.*, 1995; Guichard *et al.*, 2002). This suggests that the two proteins are expressed ubiquitously, as is Star protein in stage 6–10 oocytes (Pickup and Banerjee, 1999). Second, in an S2 cell assay, Gurken is only cleaved and secreted when cells are transfected with both Brho and Star. When transfected with Brho alone, Gurken is cleaved but not secreted, and with Star alone, Gurken is neither processed nor secreted (Ghiglione *et al.*, 2002). Because both in *squid* and *K10* mutants Gurken protein is transported all tER-Golgi units along the anterior side and at the ventral/anterior corner (our observations) and is found in the space between the oocyte and the nurse cells (Serano *et al.*, 1995; Norvell *et al.*, 1999; our observations), this suggests that at least Star and Brho are present in all tER-Golgi units, including these away from the D/A corner, and act in the processing and transport of Gurken. Along the same line, Star, Cornichon, and Brho are also likely localized to the tER-Golgi units at the posterior pole, in WT stage 6–7 and stage 9–10 *merlin* oocytes where Gurken protein is synthesized, transported and processed, so it signals to the posterior follicle cells. Third, it was recently published that in stage 10 germ line clone of the *sec5* exocyst complex subunit, Gurken protein was synthesized in the middle and at the posterior pole of the *Drosophila* oocyte (Murthy and Schwarz, 2004). This experiment, and those described above, shows that all tER-Golgi units have potentially the capacity of transporting and processing Gurken protein. Together, the tER-Golgi units at the D/A corner do not seemingly contain a different set of transport and processing proteins from the others.

It Is Gurken RNA Localization That Dictates the Use of the tER-Golgi Units

We show here, by using *K10*, *squid*, and *merlin* mutants, that what dictates the use of these numerous, seemingly identical and evenly distributed tER-Golgi units, is the restricted localization of *gurken* mRNA. This also could be the case for other transmembrane/secreted proteins.

gurken mRNA is localized in a restricted manner at the D/A corner (MacDougall *et al.*, 2003), where it is then anchored. The anchoring mechanism is not yet clear and is the subject of intense research, but we could envisage that an efficient recruitment of local tER-Golgi units would be achieved by anchoring the mRNA directly on their membrane. The ER was suggested as such an anchor by Saunders and Cohen (1999). Whatever the anchoring mechanism and wherever it is localized, *gurken* RNA diffuses locally (~20 μ m; this study), binds to ribosomes, is translated, and recognized by the signal recognition particle that targets it to the most proximal ER membrane, where the protein is synthesized and subsequently transported through the most adjacent tER-Golgi units.

Gurken Protein Is Efficiently Sorted from the ER, Preventing Its Diffusion

Additional mechanisms also ensure that local synthesis is followed by local polarized delivery at the D/A intercellular space, where the activity of Gurken is necessary but also needs to be restricted for proper oocyte development (van Eeden and St Johnston, 1999; Roth, 2003).

In eukaryotic cells, the ER has been shown to be continuous throughout the entire cytoplasm. FRAP experiments

on PDI-GFP-expressing oocytes as well as the use of a strong allelic combination of Cornichon have shown that the ER comprises a single lumen throughout the oocyte, including at the D/A corner. Partial diffusion of the newly synthesized Gurken in the membrane of the ER is therefore expected. Such a diffusion over long distance (>0.5 mm) within the ER has been shown for soluble proteins, such as the light and heavy chains of the immunoglobulins in frog oocytes (Colman *et al.*, 1982). However, the maximum distance over which intracellular Gurken is found is 20 μ m.

Therefore, the diffusion of Gurken is likely to be prevented by efficient sorting mechanisms. Those rely primarily on the transmembrane domain of Gurken and Cornichon. Gurken lacking its transmembrane domain diffuses in the ER in a very similar way as WT Gurken does in a strong *cornichon* mutant (Roth *et al.*, 1995; Queenan *et al.*, 1999; our observations), except for the concentration observed at the D/A corner. This could be explained by the difference in diffusion between a transmembrane protein and a luminal fragment. Nevertheless, this phenocopy suggests that Gurken binds Cornichon through its transmembrane domain. This interaction could mediate the efficient packaging of transmembrane Gurken in COPII transport vesicles. Cornichon presents homology to Erv14p, which is involved, in yeast, in the exit of the plasma membrane Axl2 transmembrane protein from the ER in COPII-coated vesicles. The interaction between Erv14p and Axl2p has been suggested to act via a novel mechanism that might be mediated by interactions of transmembrane segments (Powers and Barlowe, 2002). The binding of Gurken to Cornichon might rely on a similar mechanism, although it is not clear why a transmembrane cargo protein would need an extra transmembrane chaperone for its sorting and incorporation into COPII buds.

This is particularly intriguing because efficient export from the ER also could be mediated by motifs found in the cytoplasmic domain of Gurken. In type I transmembrane proteins such as ERGIC53 and Emp46, a doublet of phenylalanine and leucine, respectively, is important for the exit from the ER (Barlowe, 2003; Bonifacino and Glick, 2004). Both doublets are found in Gurken cytoplasmic domain (aa251 sfvpLLmlss lyvlfaavfm lrvpdyrrk qqqlhlh kqr FFvrc, our observation). The removal of the cytoplasmic domain of Gurken does not seem, though, to affect to a great extent the efficient exit of the truncated protein from the ER, perhaps because it can still interact with Cornichon. The role of the cytoplasmic domain could perhaps be unraveled in a weak *cornichon* mutant background.

BFA treatment of the WT egg chambers was expected to lead to full retention/retrieval of Gurken in the ER followed by its diffusion. However, many oocytes remained seemingly unaffected, suggesting that the binding of Gurken to its sorting receptor Cornichon locked Gurken at tER sites. When the drug treatment was performed in a weak allele of *cornichon*, Gurken could be observed diffusing in the ER, suggesting that the locking mechanism was impaired. This diffusion, however, was not as extensive as this observed in the strong *cornichon* mutant under nontreated conditions. This partial diffusion could represent this of the complex Cni^{CF5}/Gurken (two transmembrane proteins) instead of Gurken alone. Why, under BFA treatment, Cni^{CF5}/Gurken complex diffuses more readily than Cornichon/Gurken is still not understood and further work is needed to elucidate the molecular details of the BFA effect in oocytes.

Further work is needed to find out whether the polar synthesis and deposition other transmembrane proteins rely also on a pretranslational mechanism (through mRNA lo-

calization), alone or coupled to efficient protein sorting events from the ER.

Independence of tER-Golgi Units: Implication for Other Polarized Cells

All tER-Golgi units are able to work in synchrony for the transport of transmembrane proteins, of which the RNAs are not localized, such as Yolkless (Schonbaum *et al.*, 2000). However, we have shown, here, that a subset of tER-Golgi units can be recruited to perform the specific task of transporting a given transmembrane/secreted protein. This suggests that the different tER-Golgi units within a single cell can function in an uncoupled/nonsynchronous/independent manner, even though the ER is continuous. The restricted localization of transcripts is a necessary cue for imposing this uncoupling, as it has been suggested in muscle heterokaryons and hybrid myotubes (Pavlat *et al.*, 1989; Ralston and Hall, 1989), although it is not clear in these systems whether the ER is continuous.

We have here exemplified the functional uncoupling of the tER-Golgi units in *Drosophila* oocytes, and we propose that a similar mechanism also could take place for other types of highly polarized cells, such as neurons. This is suggested by series of observations, showing that RNA encoding transmembrane proteins specific for the dendrites are translated in the dendrites themselves, and not exclusively in the cell body (Mohr and Richter, 2003). It is also suggested by the immunofluorescence labeling of Golgi markers such as galactosyltransferase and GM130, in a dotted pattern along the dendrites (Horton and Ehlers, 2003), suggesting that perhaps Golgi-like structures could underlie this labeling. It is therefore possible that the mechanism we have identified here also occurs in neurons. mRNA encoding transmembrane/secreted proteins specific for the dendrites could be localized in these specialized domains and use dendritic Golgi-outposts to induce the local synthesis and transport of the proteins they encode.

Whether in mammalian cells, the multiple ER exit sites and the dozens of Golgi stacks making up the Golgi ribbon also could function in an uncoupled manner and respond to a restricted mRNA localization remains to be elucidated.

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