Development 135, 1923-1933 (2008) doi:10.1242/dev.017103

# The gradient of Gurken, a long-range morphogen, is directly regulated by Cbl-mediated endocytosis

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The asymmetric localization of *gurken* mRNA and post-translational sorting mechanisms are responsible for the polar distribution of Gurken protein in *Drosophila*. However, endocytosis of Egfr, the receptor for Gurken in the follicle cells, also plays a role in shaping the extracellular gradient of the Gurken morphogen. Previously, we have found that mutation in the *Cbl* gene caused elevated Egfr signaling along the dorsoventral axis, and resulted in dorsalization phenotypes in embryos and egg shells. Here, we report that overexpression of the Cbl long isoform significantly changed Gurken distribution. Using an HRP-Gurken fusion protein, we demonstrate that internalization of the Gurken-Egfr complex depends on the activity of *Cbl*. Increased levels of CblL promote the internalization of this complex, leading to the reduction of free ligands. The Gurken-Egfr complex trafficks through the Rab5/Rab7 associated endocytic pathway to the lysosomal degradation compartment for signaling termination. We observe endocytic Gurken not only in the dorsal but also in the ventral follicle cells, which is, to our knowledge, the first visualization of Gurken on the ventral side of egg chambers. Our results show that Gurken travels towards the lateral/posterior of the egg chamber in the absence of *Cbl*, suggesting that *Cbl* actively regulates Gurken distribution through promoting endocytosis and subsequent degradation.

**KEY WORDS: Gurken, Egfr, *Cbl*, Morphogen gradient, Endocytosis, *Drosophila***

## INTRODUCTION

Morphogens play pivotal roles during many important developmental stages to induce distinct cell fates by forming concentration gradients that provide positional information. The gradient formation is usually controlled by a restricted source, a limited half life and various mechanisms of trafficking through tissues (Entchev and Gonzalez-Gaitan, 2002; Tabata and Takei, 2004; Zhu and Scott, 2004). Cell surface molecules, such as morphogen receptors and heparan sulfate proteoglycans (HSPG) (Hacker et al., 2005), shape morphogen gradients by influencing their spreading. To understand the generation of positional information, it is important to assess how far a morphogen travels and how the morphogen gradient is maintained.

Gurken, a transforming growth factor α (TGFα) homolog, is a member of the Epidermal growth factor receptor (Egfr) ligand family. In *Drosophila*, this family includes a soluble secreted protein, Vein (Schnepp et al., 1996), and three transmembrane proteins, Keren (Krn), Spitz (Spi) and Gurken (Grk) (Neuman-Silberberg and Schupbach, 1993; Reich and Shilo, 2002; Schweitzer et al., 1995). The activation of Spi requires a proteolytical process involving Star and Rhomboid 1 (Rho). Star promotes the translocation of Spi from the endoplasmic reticulum to the Golgi apparatus where Rho, an intramembrane serine protease, cleaves Spi (Lee et al., 2001; Urban et al., 2001). Similarly, the transmembrane domain of Gurken is essential for its activity in the oocyte (Queenan et al., 1999), and *Star* and *brother of rhomboid* (*brho*; *stet* – FlyBase) are required for a cleavage process of Gurken to produce the secreted soluble active form of Gurken (Ghiglione et al., 2002).

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**Accepted 26 March 2008**

Egfr signaling activated by Gurken during oogenesis is required for axes determination in the future embryo (Roth, 2003). Gurken exhibits a striking asymmetrical localization at both RNA and protein level during middle stages of *Drosophila* oogenesis. The 5′ and 3′ untranslated regions, as well as part of the coding region of *gurken* mRNA target its localization tightly to the future dorsal anterior corner (Thio et al., 2000; Van De Bor et al., 2005) and by efficient sorting through the transitional endoplasmic reticulum (tER)-Golgi units, Gurken protein is also directionally transported to this dorsal anterior region (Herpers and Rabouille, 2004). The spatial restriction of Gurken activates the Egfr in a restricted number of follicle cells, leading to the establishment of dorsal follicle cell fates (Neuman-Silberberg and Schupbach, 1996). The further patterning of the dorsal egg shell structure is carried out by positive and negative feedback pathways of Egfr signaling (Freeman, 1998). Gurken activates the Egfr pathway to a maximum in the dorsal midline region where Rho expression is induced. Rho facilitates the activation of Spi that diffuses and activates Egfr in a border dorsal region. The active form of Spi is sequestered by the negative regulator Argos, which is expressed in the dorsal midline (Golembo et al., 1996; Klein et al., 2004). In addition, the negative regulator Pointed is also induced by high Egfr signaling at the dorsal midline (Deng and Bownes, 1997). As a consequence, the domain of high levels of Egfr signaling is split into two regions that promote dorsal appendage formation.

Whether Gurken travels to the ventral side of the egg chamber to influence the ventral follicle cell fate has never been completely resolved. Our previous examination of the expression of the Egfr target gene *pipe*, which is transcriptionally repressed by Egfr signaling, indicates that some level of Egfr activity is suppressed by *Cbl* in ventral follicle cells (Pai et al., 2000). In the absence of *Cbl*, *pipe* is repressed on the ventral side and this repression requires Gurken (Pai et al., 2000). Other studies suggested that Gurken indirectly regulates *pipe* expression through another diffusible morphogen induced by Notch signaling, which would be activated

by interaction between *mirror* (*mirr*) and *fringe* (*fng*) (Jordan et al., 2000). However, further analyses of *mirr* and *fng* mutant follicle cell clones indicated that these two genes have no effects on *pipe* expression. Moreover, the role of Spi, which is activated by *rho*, in regulating *pipe* expression has also been ruled out by examination of large *rho* mutant clones (Peri et al., 2002). These data suggest that the Pipe domain is directly defined by a long-range Gurken gradient. Based on indirect estimation through measuring the repression of *pipe* and mathematical modeling, the Gurken protein level at the ventral side of the egg chamber was estimated to be 10% of that at the dorsal side (Goentoro et al., 2006). However, Gurken was never directly detected at the ventral side of wild-type egg chambers, possibly owing to low levels and rapid turnover. We reasoned that if Gurken directly activates the Egfr in ventral follicle cells then, using better imaging approaches, Gurken should be detected in these follicle cells in which *Cbl* is required for Egfr signal attenuation.

In mammals, the Egfr is dimerized and autophosphorylated upon ligand activation, followed by recruitment of various proteins recognizing phospho-tyrosine to transduce or terminate Egfr signaling (Schlessinger, 2002). Cbl family proteins interact with the C-termini of activated Egfr molecules either indirectly through the adaptor protein Grb2 or directly through the tyrosine kinase binding (TKB) domain of Cbl (Schmidt and Dikic, 2005). The E3 ligase activity of Cbl promotes the ubiquitylation of activated Egfr while they are associated, and the Egfr-Cbl complex is then internalized by multiple routes (Schmidt and Dikic, 2005). The ubiquitylated receptor binds to Epsin family proteins, and goes through a non-clathrin-dependent, lipid-raft-dependent route (Sigismund et al., 2005). In addition, Cbl serves as an adaptor for interaction with the CIN85/endophilin complex, leading to dynamin-dependent clathrin-mediated receptor endocytosis (Dikic, 2003; Petrelli et al., 2002; Soubeyran et al., 2002). Previously, we have demonstrated that both Cbl isoforms (CblS and CblL) downregulate Egfr signaling, and that the efficiency of Egfr signaling attenuation is controlled by the levels of the CblL isoform (Pai et al., 2006). The genetic interaction with *shibire*, a homologue of dynamin (Chen et al., 1991; van der Bliek and Meyerowitz, 1991), further suggested that CblL may promote the internalization of activated Egfr to reduce signaling (Pai et al., 2006).

Here, we report the use of a HRP (horseradish peroxidase)-Grk fusion protein that allows us to directly trace the fate of Gurken protein in follicle cells during signaling. We found that the HRP-Grk fusion protein is internalized with the Egfr into follicle cells, a process that is mediated by Shibire. The Grk-Egfr complex trafficks through Rab5/7-associated endocytic pathways to the MVBs (multivesicular bodies) and the lysosome for signal termination. Gurken was visualized not only in the dorsal but also in ventral follicle cells. We document that Cbl facilitates the internalization of the Grk-Egfr complex into follicle cells using clonal analysis of loss of function mutations, as well as over-expression studies. Our results indicate that the distribution of the Gurken morphogen is controlled by the endocytosis of the Grk-Egfr complex through *Cbl*.

## MATERIALS AND METHODS

### Fly strains

The strains used include: wild-type (*Oregon-R, OreR)*; $grk^{HF}$ (Schupbach, 1987); $top^{IP02}$ and $top^{CO}$ (Clifford and Schupbach, 1994); *UAS-shi*$^{TS1}$ (Kitamoto, 2001); *UAS-GFP-Rab5* (Wucherpfennig et al., 2003); *UAS-GFP-Rab7* (Entchev et al., 2000); *UAS-nls-GFP* (Shiga et al., 1996); *EQ1* and *GR1* Gal4 lines (Queenan et al., 1997); $Cbl^{F165}$ (Pai et al., 2000); *UAS CblS-A1*; *UAS CblL-A18*; and *A12* (Pai et al., 2006). *e22c-Gal4* (Duffy et al., 1998) or *GR1-Gal4* was used to drive expression of UAS-Flipase in follicle cells.

### DNA constructs

To generate *HRP-grk*, DNA encoding HRP (Connolly et al., 1994) was inserted into the g*urken* genomic fragment, which is a 5 kb *Eco*RV fragment derived from the original rescuing fragment (Neuman-Silberberg and Schupbach, 1993) as shown in Fig. 2 of the publication by Thio et al. (Thio et al., 2000). This 5 kb fragment was subcloned into pBSKS as two separate plasmids, A5 (5′ *Eco*RV site at the beginning to *Eco*RV site in the third intron) and A6 (*Eco*RV site in the third intron to the *Eag* site at the end). The Grk fragment in A5 was first cloned into pcDNA using the *Eco*RV site, and then DNA encoding HRP was inserted into pcDNA *grk A5* using standard PCR-based cloning at the *Sac*II site. This *HRP-Grk* A5 fragment was cloned into A6 at the *Eco*RV site. DNA encoding the HRP-Grk fusion protein was then cloned into pCaSpeR4 with the *Apa*I-*Eag*I fragment and the construct was introduced in $w^{1118}$ hosts by P element-mediated transformation using standard methods.

### Antibodies

The antibodies used for immunocytochemistry are the monoclonal anti-Gurken antibody 1D12 made in mouse (Queenan et al., 1999); the goat anti-HRP antibody (Jackson ImmunoResearch, 1:250 dilution); the anti-CblL 8C4 ascites at 1:100 dilution (Pai et al., 2006); the anti-c-myc antibody 9E10 (Oncogene, at 1:50 dilution); a rabbit anti-Myc antibody (Santa Cruz Biotechnology); the rabbit anti-Egfr antibody, which was prepared according to the previously reported method (Jekely and Rorth, 2003) and used at 1:50; the Alexa Fluor 488 or 546 goat anti-mouse antibody (Molecular Probes); the Alexa Fluor 488 or 546 rabbit anti-goat antibody (Molecular Probes); and the TRITC-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch). The antibodies used for immunoelectron microscopy were goat anti-HRP (Sigma; 1:1000 dilution) and rabbit anti-goat IgG (DakoCytomation Denmark, Glostrup, Denmark). The antibodies were detected with protein A-gold conjugated with 10 nm gold (Cell Microscopy Center, Utrecht, The Netherlands).

### Immunofluorescent histological staining and in situ hybridization

To induce Myc expression, *e22cFLP/grk*$^{HF}$*HRP-grk; Cbl*$^{F165}$*FRT*$^{80B}$*/ πMFRT*$^{80B}$ adult females were subjected to heat shock for 1 hour at 37°C before dissection (Xu and Rubin, 1993). Ovaries were dissected in PBS and fixed for 20 minutes in 200 μl 4% paraformaldehyde (PF) saturated with 600 μl heptane and 0.25% NP40. Staining procedures were performed as described (Neuman-Silberberg and Schupbach, 1996) with minor modifications. To improve the penetration, ovaries were incubated with 1% TritonX-100 in PBST with 1% BSA before blocking, in which 10% normal rabbit serum or 1% BSA was used to reduce the background. To visualize actin, ovaries were incubated with 1 unit of phalloidin (Molecular Probes) for 30 minutes. For staining DNA, 0.5 μg/ml DAPI (Sigma) was added to the ovaries for 5 minutes. Egg chambers were further dissected and mounted in glycerol for examination under fluorescence confocal microscopy. The RNA hybridization procedure was carried out as described previously (Tautz and Pfeifle, 1989). A digoxigenin (Dig)-labeled RNA probe was made using the DIG RNA labeling Kit (Roche). The probe was detected with alkaline phosphatase conjugated anti-DIG antibody (1:5000 dilution, Roche) and the NBP/BCIP developing kit (Promega).

### Immunoelectron microscopy and quantification

Ovaries were dissected and fixed in a mixture of 0.2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer for 3-4 hours. The individual egg chambers were embedded in 12% gelatin. The blocks were trimmed to size, immersed in PVP/sucrose (15%/1.7M) at 4°C overnight, mounted on aluminum pins in the proper orientation and stored in liquid nitrogen. Frozen sections were prepared by using an ultracryomicrotome (Reichert Ultracut S/Reichert FCS, Leica; Vienna, Austria). To obtain ultrathin sections that included the oocyte nucleus, 0.5 μm semithin sections were first cut, at –60°C, and examined with a light microscope after staining with 0.1% Toluidine Blue in 1% boric acid. When the nucleus was reached, the cutting temperature was lowered to –100°C and section thickness switched to 55 nm. Ultrathin sections were picked up with a drop of an equal volume mixture of methylcellulose (2%)/sucrose (2.3 M). The ultracryomicrotomy and protocol used for immunolabeling has been described in detail (Liou et al., 1996). Samples were viewed under a JOEL

JEM-1230 transmission electron microscope. To compare the endocytic activity of follicle cells at different zones, the volume density of MVB/lysosomes per follicle cell profile was estimated by the point-counting method (Griffiths, 1993). Only those cell profiles exhibiting MVB/lysosomes were graphed. Sampling was from 12 different grids (nine *OreR* and three $grk^{HF}/grk^{HF}$) from seven egg chambers (five *OreR* and two $grk^{HF}/grk^{HF}$). Cell profiles analyzed (pn) are indicated in the figure. Total areas quantified were 3227.23 $\mu m^2$ for *OreR* and 693.39 $\mu m^2$ for $grk^{HF}/grk^{HF}$.

## RESULTS

### The subcellular localization of Gurken in posterior follicle cells during early oogenesis stages

The distribution of Gurken is dynamic in the oocyte during oogenesis. At stage 7, when the Gurken signal is required to induce a posterior cell fate in the adjacent follicle cells through Egfr activation, Gurken is mainly localized in the entire cytoplasm of the oocyte. A punctate distribution of Gurken in the apical side of posterior follicle cells can be easily detected with high magnification, as shown in Fig. 1A,B and previous reports (Ghiglione et al., 2002; Queenan et al., 1999). These punctate signals in the posterior follicle cells are very likely to be the Grk-Egfr complex in endocytic vesicles after the complex is internalized.

To test this possibility, we examined the effect of a dominant-negative form of Shibire [*UAS-shi*$^{ts}$ (Kitamoto, 2001)] on the distribution of Gurken. Under a non-permissive temperature, these punctate signals in follicle cells were dramatically reduced (Fig. 1C,D), suggesting that Gurken accumulates in posterior follicle cells through a Dynamin-dependent process. The localization of Gurken in endocytic vesicles was further confirmed by the colocalization with a *GR1-Gal4* driven expression of the early endosomal marker (GFP-Rab5) (Wucherpfennig et al., 2003) and a late endosomal marker (GFP-Rab7) (Entchev et al., 2000), respectively (Fig. 1E-H′). These data indicate that Gurken is internalized into posterior follicle cells through the Shibire and Rab5/7-associated endocytic pathway at the stage when the posterior follicle cell fate is defined.

After stage 8, the oocyte nucleus moves to the anterior of the egg chamber, and Gurken is localized around the oocyte nucleus. During this stage, the Gurken signal in follicle cells was only detected in 17% ($n$=24) of egg chambers, and the expression was restricted to a few follicle cells adjacent to the nucleus of oocyte (Fig. 1I-L). The punctate signals within individual follicle cells possibly correspond to the endocytic compartments, similar to that which was observed in the posterior follicle cells.

### An active HRP-Grk fusion protein

Previous studies have successfully used HRP-Boss (Sunio et al., 1999) and HRP-Wingless fusion proteins (Dubois et al., 2001) to study ligand distribution in endocytic trafficking. To visualize the endocytic trafficking of Gurken during stages 8 to 10, we made an HRP-Grk fusion to use as a tracer because HRP is relatively stable within the destructive environment of the endosome and lysosomal compartments. As 5′ and 3′ UTR, as well as part of the coding region of *gurken*, are important for the correct localization of *gurken* RNA in *Drosophila* oogenesis (Thio et al., 2000; Van De Bor et al., 2005), HRP was inserted into exon 3 of the *gurken* genomic sequence, between the signal peptide and the EGF repeat (Fig. 2A). We expected that this fusion protein, as driven by the endogenous

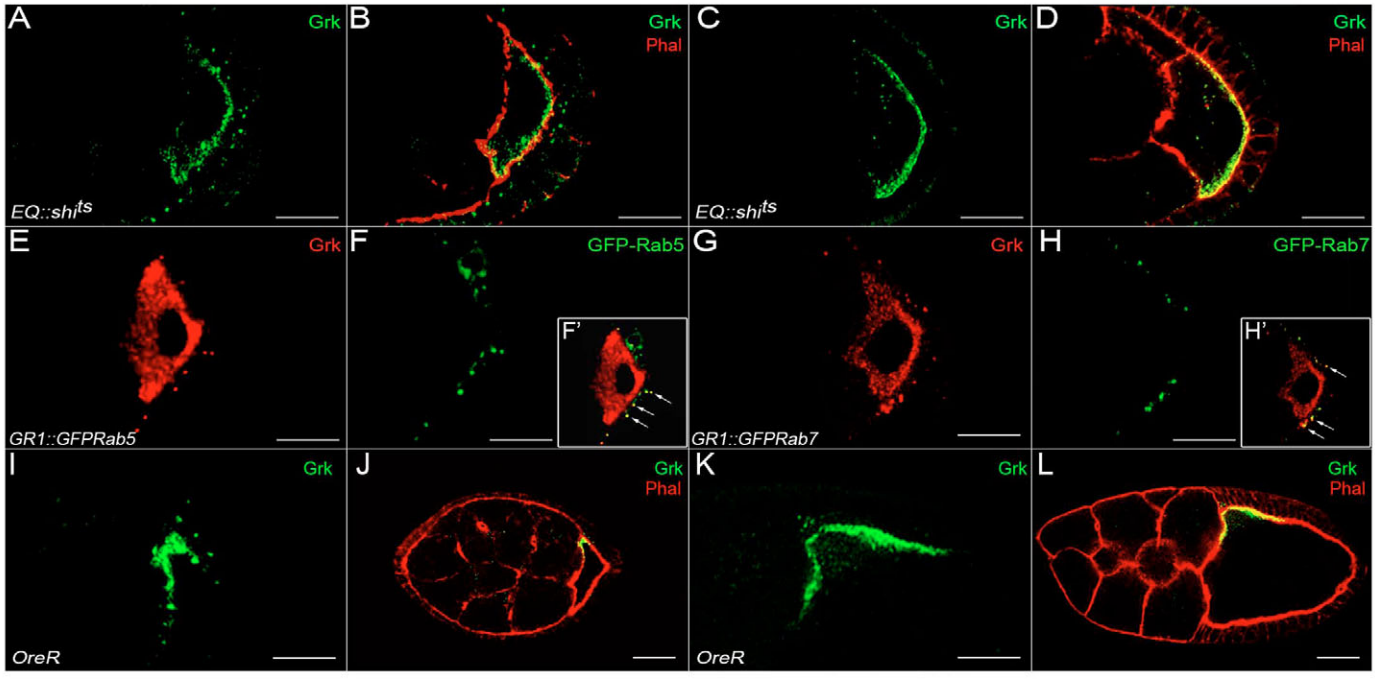


**Fig. 1. Subcellular localization of Gurken in follicle cells.** Anterior of the egg chamber is towards the left and dorsal side faces upwards. (**A-D**) The localization of Gurken in the posterior follicle cells during early oogenesis stages was dependent on *shibire*. Gurken was labeled with antibody (green). The oocyte and follicle cell cortex were visualized by phalloidin staining (red). Gurken was easily detected in the posterior follicle cells around the oocyte at permissive temperature in *EQ1::shi*$^{ts}$ egg chambers (A,B). These punctate signals were significantly reduced at a non-permissive temperature (C,D). (**E-H**) Gurken (red) colocalized with early endosomes (labeled with GFP-Rab5, E,F), and late endosomes (labeled with GFP-Rab7, G,H) in posterior follicle cells. Arrows indicate colocalization in the merged images (F′,H′). (**I-L**) During middle oogenesis when Gurken (green) shows an asymmetrical localization in the oocyte, little Gurken could be detected in dorsal follicle cells. At stage 8 (I,J) and 9 (K,L), Gurken signals in dorsal follicle cells were detected in about 17% of the egg chambers. Scale bars: 10 μm in A-I; 20 μm in J,K; 40 μm in L.

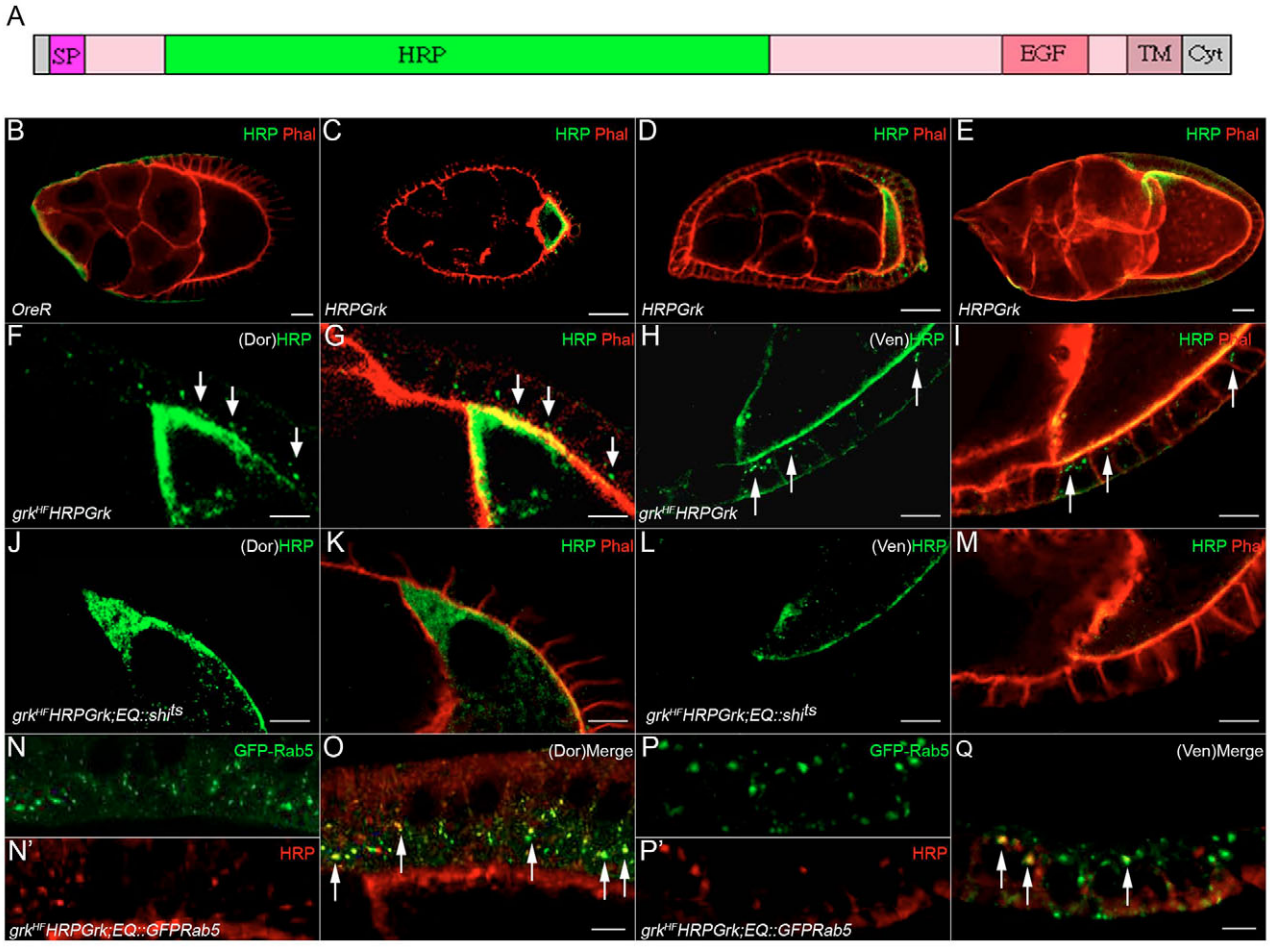


**Fig. 2. Expression pattern of the HRP-Grk fusion protein.** (**A**) The schematic of the HRP-Grk fusion protein. SP, signal peptide; EGF, EGF repeat motif; TM, transmembrane domain; Cyt, cytoplasmic domain. (**B-M**) The localization of HRP-Grk was detected by the anti-HRP antibody (green), and the egg chamber organization was identified by phalloidin staining (red). The wild-type egg chambers served as a negative control (B). HRP staining in the oocyte at stage 7 (C), stage 8 (D) and stage 9 (E) showed similar patterns to endogenous Gurken staining. The punctate HRP signal was detected not only in the dorsal (arrows in F and G) follicle cells but also in the ventral follicle cells (arrows in H,I). These punctate signals were markedly reduced at a non-permissive temperature in *shibire* mutant background (J-M). (**N-Q**) Some HRP signal (red) in follicle cells colocalized with an early endosomal marker, GFP-Rab5, in the dorsal (N,N′,O) and ventral (P,P′,Q) follicle cells (arrows indicate punctate signals that overlapped in merged images). Anterior of the egg chamber is towards the left and dorsal side faces upwards. Scale bars: 20 μm in B-E; 10 μm in F-Q.

*gurken* promoter, could mimic the endogenous expression and trafficking of the Gurken protein, and would still be detected after processing through endocytosis.

Several genetic and biological assays were performed to test whether the HRP-Grk fusion protein was correctly localized and could activate the Egfr pathway. HRP-Grk was homogenously expressed in the oocyte during early stages (Fig. 2C), and localized correctly to the dorsal-anterior corner of the oocyte during mid-stages (Fig. 2D,E). Similar to endogenous Gurken, HRP-Grk was not detected after stage 10B (data not shown). Therefore, the HRP insertion in the fusion protein did not alter the expression and localization of the Gurken protein. Furthermore, we tested the biological function of HRP-Grk in rescuing the phenotypes of *grk* mutants. The heterozygous null mutant females of $grk^{HF}$ laid mildly ventralized eggs (about 54%) and homozygous mutant females laid 100% eggs with strong ventralized egg shells. These phenotypes could be partially rescued by the *HRP-grk* transgene in seven independently derived lines, with the line *HRP-grk-5* giving the highest rescuing ability as *HRP-grk-5B* almost completely reverted the heterozygous mutant phenotype to that of the wild-type (99%) (Table 1). In addition, the rescue ability showed dose dependence, two copies of this transgene rescued to a higher degree than one copy when tested in the homozygous $grk^{HF}$ mutant. These results demonstrate that the HRP-Grk fusion protein can activate Egfr signaling similarly to endogenous Gurken, and that the expression level is not saturated.

## The Grk-Egfr complex is detected in the endocytic pathway of both dorsal and ventral follicle cells

As expected, the HRP-Grk provided higher detection sensitivity than did Gurken. The double staining with anti-Grk and anti-HRP antibodies on HRP-Grk expressing egg chamber showed that nearly all Grk signal in the oocyte or follicle cells overlapped with HRP signal, but some HRP signal in follicle cells was Grk negative (see Fig. S1 in the supplementary material). Furthermore, in ventral follicle cells, no signal was detected by the anti-Grk antibody, whereas punctate HRP signals were obvious. The data indicate that the difference between Gurken and HRP-Grk is in detection

**Table 1. *HRP-grk* transgene rescued the ventralized egg shell phenotype in *grk* mutants**

| Genotype | Phenotype  * | | | | *n* |
|---|---|---|---|---|---|
| *OreR* | – | – | – | 100% | 193 |
| $grk^{HF}$/+ | – | – | 54% | 46% | 320 |
| $grk^{HF}/grk^{HF}$ | 100% | – | – | – | 49 |
| $grk^{HF}$*HRP-grk-5/CyO* | – | – | 1% | 99% | 116 |
| $grk^{HF}$*HRP-grk-5/*$grk^{HF}$ | – | 87% | 13% | – | 47 |
| $grk^{HF}$*HRP-grk-5/*$grk^{HF}$*HRP-grk-5* | – | – | 38% | 62% | 93 |
| $grk^{HF}$*/Cyo;HRP-grk-2/Ser* | – | – | 3% | 97% | 179 |
| $grk^{HF}$*/Cyo;HRP-grk-8/Ser* | – | – | 2% | 98% | 262 |

*The egg-shell phenotypes are arranged in an order from strong ventralization to wild type (left to right). In the most severe *grk* mutant phenotype, no dorsal appendage (DA) is formed (first column). The *grk* mutant containing one copy of the *HRP-grk* transgene shows a phenotype with one fused DA (second column). The weakly ventralized egg shell has two DAs fused at the base (third column).

sensitivity rather than distribution. The frequencies of detecting HRP-Grk in follicle cells using an anti-HRP antibody during stages 8 to 9 were 88.6% ($n$=53 egg chambers) at the dorsal side and 69% ($n$=55) at the ventral side (Fig. 2F-I). As seen in the longitudinal section of confocal images of stage 8 to 9 egg chambers, HRP-Grk was not only detected in six to ten (the 1st to 10th main body follicle cell counting from anterior to posterior) follicle cells overlying the oocyte nucleus (Fig. 2F,G), but also in four to six follicle cells at the opposite ventral side (Fig. 2H,I). This was the first time that Gurken was detected in ventral follicle cells at a stage when Gurken is asymmetrically localized at the anterior corner of the oocyte. This is consistent with theoretical and genetic evidences provided previously (Goentoro et al., 2006; Pai et al., 2000; Peri et al., 2002), which indicated that some level of Egfr activity in ventral follicle cells is activated by Gurken.

To test whether the internalization of HRP-Grk in dorsal and ventral follicle cells is Dynamin dependent, HRP signal was examined in the temperature-sensitive and dominant-negative *shibire* mutant *UAS-*$shi^{ts}$. The punctate HRP signals were dramatically reduced both in dorsal and ventral follicle cells during stage eight to nine at non-permissive temperature (Fig. 2J-M), suggesting that Dynamin has a crucial role in the internalization process of Gurken. To follow the trafficking route of HRP-Grk, we marked early endosomes with *UAS-GFP-Rab5* driven by *GR1-Gal4*, expressed in all follicle cells, starting from the germarium. Some HRP-Grk signals were colocalized with Rab5 both in dorsal and ventral follicle cells (59%, $n$=44, Fig. 2N-Q). Similarly, colocalization of HRP-Grk and GFP-Rab7 (36%, $n$=33), a late endosomal marker, was also detected (data not shown).

We then asked whether the internalization of Gurken is dependent on its interaction with Egfr. We generated an anti-Egfr antibody to monitor colocalization of HRP-Grk and Egfr. The specificity of this antibody was tested in mosaic egg chambers with Egfr mutant clones (Fig. 3A-C). Indeed, HRP-Grk and Egfr were colocalized (62%, $n$=39) in dorsal (Fig. 3D-F) and ventral (data not shown) follicle cells as revealed by double staining with anti-HRP and anti-Egfr antibodies. We then generated Egfr mutant clones in the follicular epithelium to test whether HRP signal in follicle cells is dependent on the presence of Egfr, and hence corresponds to the HRP-Grk-Egfr complex internalized into signal receiving cells. Two *torpedo* (*top*)/*Egfr* null mutant alleles, Egfr[CO] and Egfr[IP02], were tested. The molecular information of Egfr[IP02] suggests that this mutated Egfr has no cytoplasmic domain for signaling (Clifford and Schupbach, 1994). In the Egfr mutant follicle cells, marked by the absence of GFP signal, the HRP signal was significantly reduced or entirely absent (Fig. 3G-I). The fact that the HRP signal in follicle cells is Egfr-dependent suggests that Gurken binding triggers the entry of the ligand-receptor complex into follicle cells. We also found that, once inside the cells, Egfr localizes to Rab5 positive vesicles near the apical region and to Rab7-positive vesicles in the region between the apical membrane and the nucleus, respectively (Fig. 3J-O). Given that internalized Gurken in both dorsal and ventral follicle cells depends on a functional Egfr, these data indicate that Egfr is activated by Gurken not only in dorsal but also in ventral follicle cells. The results also show that the ligand-receptor complex is mainly internalized through the Dynamin- and Rab5/7-associated endocytic pathway at the stage when Gurken defines the dorsoventral cell fate.

## The Grk-Egfr complex is degraded in the lysosomal compartment

To visualize the subcellular localization of HRP-Grk, we applied immuno-electron microscopy using an anti-HRP antibody. Herpers and Rabouille (Herpers and Rabouille, 2004) studied the exocytic pathway of Gurken in stage 9 *Drosophila* oocytes by immuno-electron microscopy using the Gurken antibody, and demonstrated that the Gurken protein is directionally transported through the tER-Golgi units at the dorsal-anterior corner of the oocyte. We first tested whether HRP-Grk trafficks through the same exocytic pathway. Consistent with their observation, we found that HRP-Grk positive tER-Golgi units were significantly concentrated in the D/A corner of the ooplasm (7.7~16.9 gold/units), whereas the labeling density in tER-Golgi units located in the ventral or posterior of the ooplasm was much weaker (0.3~0.8 gold/units) (Fig. 4C-E and data not show). Gurken protein gradually reached the highest peak at middle stage 9 in the tER-Golgi units at the dorsal anterior corner, and declined at late stage 9 (Fig. 4E). This expression pattern corresponds well with the pattern detected by immunostaining with the Gurken antibody, in which no Gurken signal can be detected after stage 10B (Neuman-Silberberg and Schupbach, 1996). We also checked the labeling in the space between the oocyte and the overlying follicle cells to examine the extracellular gradients of Gurken. However, a strong labeling even in wild-type egg chambers not expressing HRP-Grk was detected, which might result from trapped gold particles within this region (data not shown).

We then examined the fate of the Grk-Egfr complex in the follicle cells at stage 9. Gold particles in the follicle cells corresponding to Gurken in our cryosectioned fixed stage 9 egg chambers showed a distinct pattern. Except for some nonspecific binding to mitochondria, major gold particles were located in unidentified

small vesicles, the MVB, and the lysosome in both dorsal and ventral follicle cells (Fig. 4F,G). We previously demonstrated that the internalization of the Grk-Egfr complex plays a major role in terminating Egfr signaling, as dorsalized egg shell phenotypes resulting from elevated Egfr signaling were observed when *shibire* activity was blocked (Pai et al., 2006). Taken together, our data indicate that the internalized Gurken travels through the early and late endosomes, and is targeted to degradation in the MVB/lysosome compartment. Consistent with our immunostaining result, this signaling attenuation process occurs both in the dorsal and ventral follicle cells, again indicating that Gurken has reached the ventral side of the egg chamber to activate the Egfr at ventral follicle cell plasma membrane. Interestingly, we noted that after early stage 9, the lateral follicle cells contained a larger lysosomal area than did the dorsal/ventral follicle cells (Fig. 4H-J). This difference was not observed in a *gurken* mutant background, indicating an early response to Egfr signaling in these cells (Fig. 4K).

## The role of Cbl in regulation of Gurken distribution

Consistent with the role of *Cbl* as a negative regulator in Egfr signaling, overexpression of CblL results in phenotypes that are characteristic of reduction of Egfr signaling. Furthermore, overexpression of CblL suppressed the effect of reduced internalization of Egfr, suggesting that CblL may promote internalization of activated receptors (Pai et al., 2006). To further investigate the precise mechanisms by which Cbl regulates Egfr signaling, we examined the pattern of Gurken distribution in egg chambers with overexpression of CblL driven by *GR1-Gal4*. When analyzing Gurken by immunofluorescence, it is not possible to distinguish between Gurken protein that is still at the oocyte membrane versus Gurken protein that is extracellular or bound to the follicle cell surface. In egg chambers with over-expressed CblL, at this level of resolution, we found that Gurken was markedly reduced in late stage 9 egg chambers when Gurken should have reached its highest expression peak. This reduction was seen in more than 40% ($n$=190) of egg chambers with CblL overexpression (Fig. 5C,D). Some variation of Gurken immunostaining was seen in about 10% of control wild-type egg chambers ($n$=96) (Fig. 5D). The reduction occurred only at the protein level, while the mRNA was not changed, as indicated by an in situ hybridization assay (data not shown). By contrast, overexpression of CblS had much more subtle effects (Fig. 5B,D). Furthermore, in contrast to the reduction in the intercellular and membrane region, the internal punctate HRP signals were dramatically increased in follicle cells with medium overexpression of CblL, but were not detectable in cells with high expression levels of CblL (Fig. 5E-H). EQ1 and *GR1-Gal4* driven expression in the follicle cells is somewhat patchy, resulting in follicle cells with both higher and somewhat lower expression levels, allowing a direct comparison of the effects of higher versus

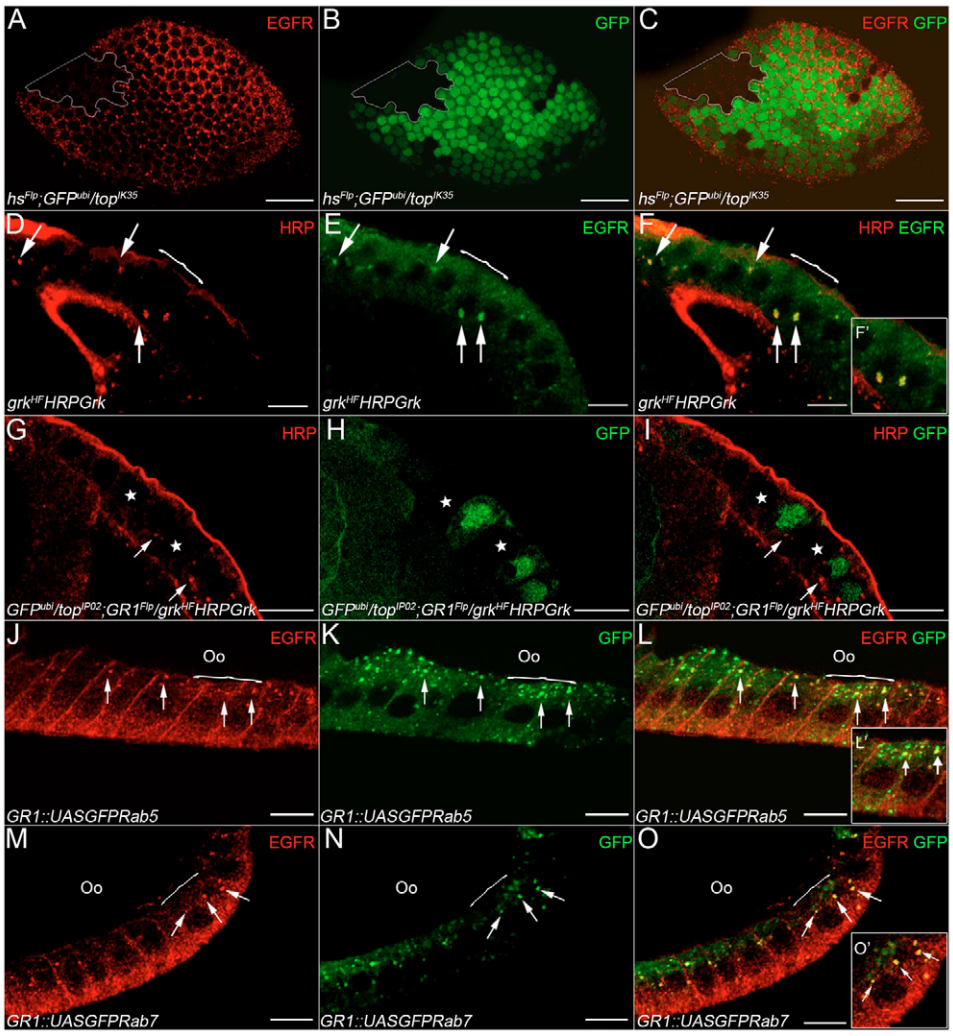


**Fig. 3. The Grk-Egfr complex is internalized in follicle cells through Rab5-Rab7 endocytic pathways.** (**A-C**) Clonal analysis of mosaic egg chambers shows that the anti-Egfr antibody staining (red) is specific as it is absent from the mutant cells. (**D-F'**) HRP-Grk (red) was colocalized with Egfr (green) in dorsal follicle cells. (**G-I**) Punctate HRP signals (red) were much less abundant in the *top/Egfr* mutant follicle cells (star indicates non-GFP cells) compared with adjacent normal cells (arrows indicate punctate HRP signals in wild-type cells). (**J-O**) Internalized Egfr (red) was colocalized with Rab5 (J-L) and Rab7 (M-O) in the follicle cells. Oo, oocyte; arrows indicate punctate signals that overlapped in merged images (D-F,J-L,M-O); the bracket area was enlarged in F′,L′,O′. Scale bars: 10 μm.

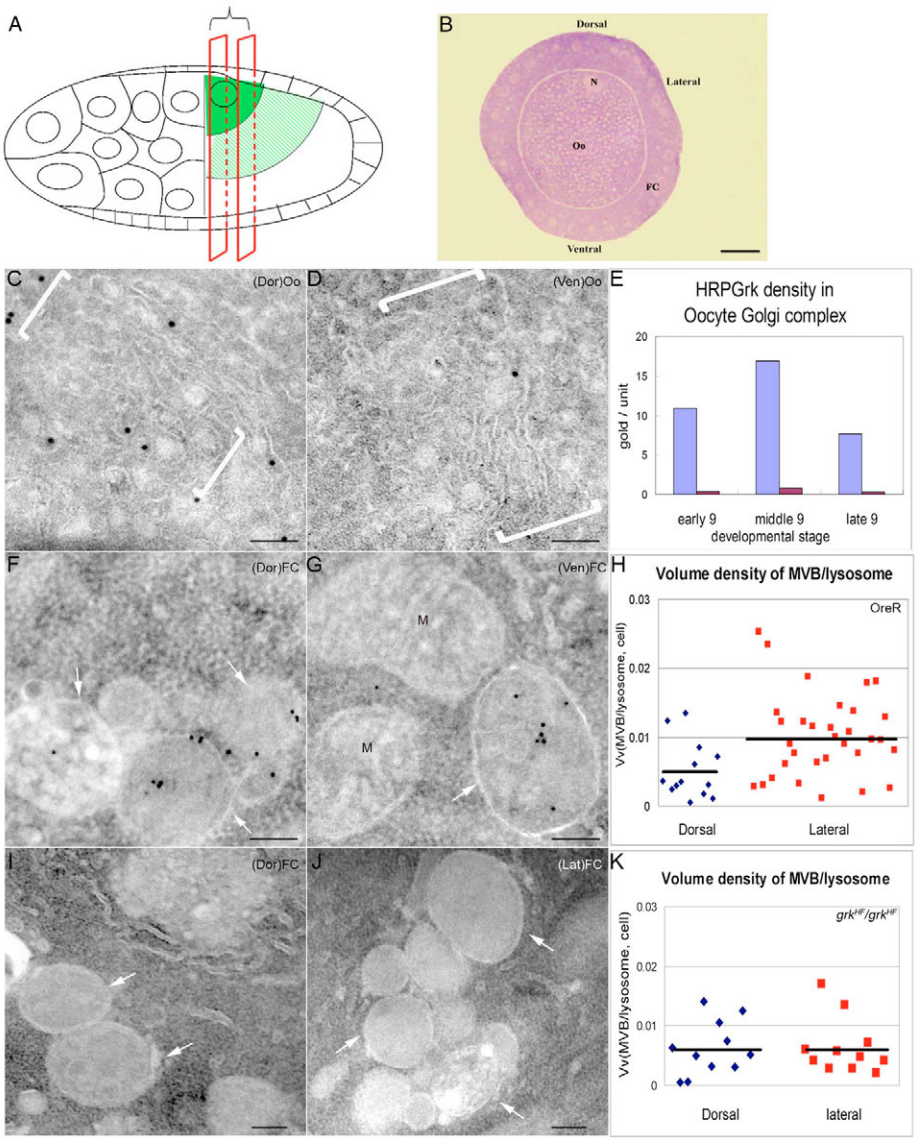


**Fig. 4. The degradation of HRP-Grk in follicle cells.** (**A**) Schematic representation of our sectioning strategy. The data shown below were all taken from cross-section profiles of stage 9 egg chambers that included oocyte nucleus. (**B**) Micrograph of a 1 μm cryosection. N, oocyte nucleus; Oo, oocyte; FC, follicle cells. (**C-G**) Electron micrographs of stage 9 oocytes immunolabeled with anti-HRP (10 nm gold particles). The labeling density of HRP-Grk at TGNs (brackets) is higher on the dorsal side (C) than on the ventral side (D) of the egg. (E) Quantitation of HRP-Grk labeling per TGN unit in the oocyte. $n$=3~10. Among the organelles, the single-membrane delimited MVB/lysosome (arrows) exhibited the most concentrated labeling, both in the dorsal (F) and the ventral (G) follicular cells. M, mitochondria. (**H-J**) The MVB/lysosomes in follicle cells of late stage 9 OreR egg chamber. Electron micrographs of MVB/lysosomes in the dorsal (I) and lateral (J) follicular cells. (H) Semi-quantification of the volume density of MVB/lysosomes in follicular cells. The average density ratio (bar) in dorsal, and lateral follicle cells is 0.0060 and 0.0109, respectively. The difference between the dorsal and lateral follicle cells is meaningful in the nonparametric Wilcoxon test (two-tailed, $P$=0.007). (**K**) Semi-quantification of the volume density of MVB/lysosomes in follicle cells of late stage 9 $grk^{HF}/grk^{HF}$ egg chambers. The average density ratios (bar) for dorsal and lateral are 0.0062 and 0.0065, respectively. Scale bars: 20 μm in B; 100 nm in C,D,F,G,I,J.

intermediate levels of CblL (see Fig. S2 in the supplementary material). This suggests that in cells with high levels of CblL the receptor-ligand complexes may be undergoing very rapid trafficking and degradation. In the CblL overexpressing follicle cells, colocalization of HRP-Grk punctate signals with Rab5 (41%, $n$=27, Fig. 5I), Rab7 (67%, $n$=21, Fig. 5J), and Egfr (51%, $n$=41, Fig. 5K) were detected. Taken together, these results suggest that more Grk-Egfr complexes are in the follicle cells with the help of CblL than that in wild-type cells without CblL overexpression. This leads to a reduction of Gurken outside the follicle cells and an increase of Gurken inside the follicle cells. As a consequence, the promotion of Grk-Egfr endocytosis by CblL can change the overall distribution of the Gurken morphogen. In summary, Cbl negatively regulates Egfr signaling by facilitating the internalization and degradation of Grk-Egfr complex and thus helps to shape the pattern of Gurken protein expression.

We next tested the effect of *Cbl* loss-of-function on Gurken expression and distribution. Unlike the significant changes caused by CblL overexpression, there was no globally detectable difference in the overall Gurken expression pattern in egg chambers containing *Cbl* mutant follicle cell clones (Fig. 6A,B). But a strong reduction of the internalized Grk-Egfr complex in the *Cbl* mutant follicle cells was observed in dorsal (Fig. 6F,G), ventral (Fig. 6H) and posterior regions (Fig. 6C,D) of egg chambers, indicating that the internalization of Grk-Egfr complexes autonomously requires functional *Cbl*. Interestingly, at a local level, high levels of the Grk-Egfr complex were detected in wild-type cells adjacent to *Cbl* mutant cells (Fig. 6F,G). Furthermore, the HRP-Grk signal was detected even in the 11th-12th main body follicle cells when the *Cbl* mutant clone was located in the dorsal-anterior corner, whereas normally no HRP-Grk signal was detected in follicle cells located more posteriorly than the 10th follicle cell (Fig. 6E). These results very strongly suggest that Gurken is readily diffusible and travels further towards the posterior region, when the Grk-Egfr complex is not efficiently internalized in anterior follicle cells.

## DISCUSSION

### Gurken diffuses from the dorsal side to the ventral side of egg chambers

In the *Drosophila* ovary, different levels of Egfr signaling are crucial for axis establishment of the egg and the future embryo. The asymmetrically localized Gurken has been suggested to provide

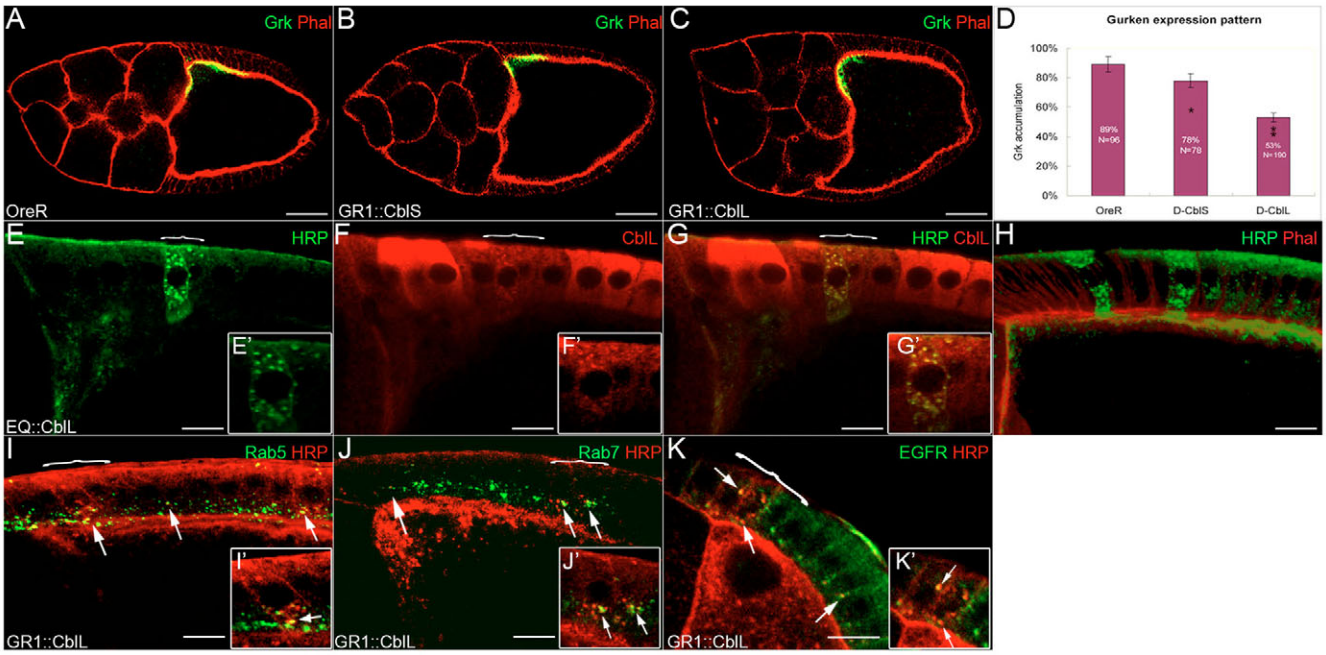


**Fig. 5. Overexpression of CblL altered Gurken distribution.** Anterior of the egg chamber is towards the left and dorsal side faces upwards. (**A-C**) Gurken expression pattern (green) at late stage 9 egg chambers was altered when Cbl was overexpressed. In wild-type egg chambers, Gurken accumulated in the dorsal anterior corner (A). Slightly reduced expression of Gurken was detected in egg chambers with overexpression of CblS driven by *GR1-Gal4* (B). Significantly reduced expression of total Gurken in egg chamber in which CblL was overexpressed (C). (**D**) The percentage of late stage 9 egg chambers that had normal Gurken protein expression. $*P>0.05$; $**P<0.01$. (**E-H**) The increased HRP-Grk signal (green) in dorsal follicle cells (E) was colocalized with CblL, which was ectopically expressed by *EQ-Gal4* and stained with 8C4 antibody (F, red). (H) The composite image of several confocal sections of an egg chamber with an *HRP-grk* transgene and CblL overexpression. (**I-K**) Some of these HRP-Grk signals were also colocalized with Rab5 (I), Rab7 (J) and Egfr (K). Arrows indicate punctate signals that overlapped in merged images. The bracket area is enlarged in E′,F′,G′,I′,J′,K′. Scale bars: 40 μm in A-C; 20 μm in E-J; 10 μm in K.

different levels of ligand, leading to various levels of Egfr activation. Previous studies had suggested that Gurken acts as a free soluble ligand released by the oocyte after proteolytic processing in the Golgi compartment (Ghiglione et al., 2002). However, a direct examination of the Gurken gradient has never been possible, as it has been very difficult to distinguish the distribution of Gurken in the oocyte, in the extracellular space between the oocyte and the follicle cells or on the follicle cell membrane with the resolution of confocal microscopy in immunostaining assays. We tried to examine Gurken through TEM, but non-specific binding of the anti-Gurken antibody in the space between the oocyte and the follicle cell made this impossible. Nevertheless, it is important to know how far Gurken can travel in the egg chamber to evaluate whether all ranges of Egfr activation are determined by one ligand, Gurken, or whether they might also be affected by other signals. We therefore took an alternate approach and examined the ligand-receptor complex in the signal-receiving cells.

The fate of Gurken after binding to its receptor has not been previously analyzed. We fused Gurken to HRP, which allowed a more sensitive detection for Gurken after its internalization into the follicle cells (see Fig. S1 in the supplementary material; Figs 1 and 2). The presence of the cytosolic HRP positive particles depends on the presence and function of Egfr, indicating that Gurken is internalized with the Egfr as a complex. Our results with *shibire* mutants further demonstrate that Dynamin is required for this process (Fig. 2J-M and Fig. 3G-I). Interestingly, we never saw clathrin-coated pits with or without HRP in follicle cells, whereas in the oocyte, clathrin-coated pits were easily detected (data not shown). It is possible that clathrin-dependent internalization is not a major pathway for the Grk-Egfr complex. Both Gurken and Egfr are colocalized with Rab5 and Rab7 (Fig. 2N-Q, Fig. 3J-O), suggesting that the Grk-Egfr complex takes this endocytic pathway to the MVB/lysosome compartment, where signaling is terminated and Gurken is degraded. Accumulating evidence suggests that receptor signaling can occur in different cellular compartments (Hoeller et al., 2005), and Egfr signaling in the late endosome has been shown to mediate through a p14/MP1 scaffold (Teis et al., 2002). However, in *Drosophila* follicle cells, the function of internalization of the Grk-Egfr complex is mainly to terminate signaling, as blocking endocytosis by interfering with Dynamin function results in an excess Egfr signaling phenotype (Pai et al., 2006). In other words, the Egfr signaling mainly occurs on the cytoplasmic membrane in follicle cells.

Our data showed that a significant amount of HRP-Grk was detected in ventral follicle cells by immunostaining and immunoelectron microscopy using an anti-HRP antibody. Direct secretion of Gurken from the ventral surface of the oocyte to the ventral follicle cells is very unlikely, given results from tER studies carried out by both Rabouille and colleagues, as well as by us. Furthermore, the autonomous effect of *Cbl* clones on the vesicular signal of HRP-Grk in dorsal and ventral follicle cells suggest that the major spreading of Gurken in the egg chamber was not achieved through transcytosis (Fig. 6C-H). Our results are much more compatible with a mechanism where Gurken diffuses as a free secreted form or in a complex with some extracellular molecule such as HSPG (Belenkaya et al., 2004; Hacker et al., 2005), and

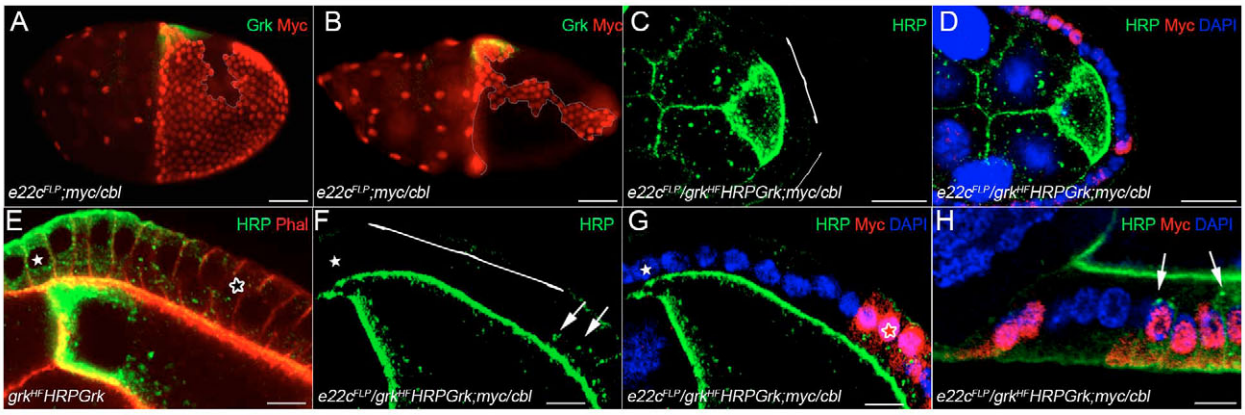


**Fig. 6. The Gurken internalization was blocked in *Cbl* mutant follicle cells.** Anterior of the egg chamber is towards the left and dorsal side faces upwards. Wild-type cells were marked by anti-Myc antibody (red). Mosaic egg chamber containing *Cbl* mutant clones and an *HRP-grk* transgene were obtained from females of the genotype *e22cFLP/grk$^{HF}$HRP-grk; Cbl$^{F165}$FRT$^{80B}$/MFRT$^{80B}$*. (A,B) Gurken expression (green) pattern was not markedly altered in egg chambers containing *Cbl* mutant follicle cell clones. (C,D,F-H) Punctate HRP-Grk signal was significantly reduced in the posterior (C,D), dorsal (F,G) and ventral (H) follicle cells, in which *Cbl* was homozygous mutant (areas indicated by brackets). When a large *Cbl* mutant clone was present in the dorsal region, the punctate HRP-Grk signal could be detected in follicle cells situated more posteriorly (F, arrows indicate punctate HRP signals; G, open stars indicate the 10th follicle cells) than in wild-type egg chambers (E). The 1st main body follicle cell was the one overlying the border between nurse cells and the oocyte (indicated by a star). Scale bars: 40 μm in A,B; 20 μm in C,D; 10 μm in E-H.

distributes to the dorsal and ventral side in the space between the oocyte and follicle cells. This interpretation is also consistent with a quantification model of the Gurken morphogen in which 10% of Gurken was predicted to appear on the ventral side of egg chambers compared with dorsal levels (Goentoro et al., 2006). Taken together, our data demonstrate that Gurken acts as a long-range morphogen, which directly determines the fates of ventral follicle cells.

## Endocytosis of the Grk-Egfr complex through Cbl shapes the Gurken morphogen gradient

Morphogen gradients are controlled by several mechanisms, such as the level of receptors and the rate of endocytosis (Lecuit and Cohen, 1998; Teleman et al., 2001). Goentoro and colleagues have shown that overexpression of Egfr in the follicle cells traps Gurken near the dorsal anterior corner and prevents its diffusion, leading to an expansion of Pipe expression (Goentoro et al., 2006). This finding suggests that in the wild-type situation, most Egfr is bound with Gurken in dorsal follicle cells, and excess free Gurken diffuses to the lateral/ventral side of egg chambers to form the gradient.

Here, we report that endocytosis also plays a crucial role in shaping the gradient of the Gurken protein, which is similar to observations for Wingless and Fgf8 (Dubois et al., 2001; Scholpp and Brand, 2004). Dubois and colleagues had shown that endocytosis regulates the asymmetry of the Wingless gradient using HRP-Wingless to monitor Wingless degradation. It has been well established that Cbl acts as an endocytic adaptor to mediate the internalization of Egfr in mammals (Rubin et al., 2005; Schmidt and Dikic, 2005). Our previous data showed that overexpression of CblL resulted in the reduction of Egfr signaling in eggshell patterning and repression of its target gene, *argos* (Pai et al., 2006). We demonstrate here that Cbl facilitates the endocytosis of the Grk-Egfr complex into follicle cells based on our clonal analysis of *Cbl* mutant follicle cells (Fig. 6C-H). Very strikingly, neighboring wild-type cells contained more punctate HRP-Grk signals (Fig. 6E,F), indicating that more Gurken was available to these wild-type cells. This result suggests that larger quantities of free Gurken can travel towards the posterior when the Grk-Egfr complex cannot efficiently enter the dorsal anterior cells in the absence of *Cbl*. However, this subtle change in Gurken distribution cannot be revealed through examining the overall Gurken gradient by immunostaining (Fig. 6A). Conversely, more Grk-Egfr complexes were removed from the membrane to the Rab5/7-associated endocytic pathway in follicle cells with over-expressed CblL when compared with those in wild-type cells, and under these conditions, Gurken diffusion was limited (Fig. 5C,H-K). This observation indicates that CblL serves as a rate-determining component of the endocytic pathway. Increased levels of CblL effectively promote the endocytosis of Egfr. Furthermore, the significant reduction of Gurken protein in egg chambers with CblL overexpression at stage 9 suggests that internalization of the Grk-Egfr complex may lead to a rapid recycling of Egfr on the cytoplasmic membrane of follicle cells and thus allow more binding to Gurken. In conclusion, we demonstrate here that increasing the rate of endocytosis by overexpression of CblL can modify the Gurken distribution.

Interestingly, we noticed that the area of the lysosome and MVB vesicle in the lateral follicle cells was bigger than that in the dorsal or ventral follicle cells in wild-type egg chambers, suggesting that the endocytic activity was higher in the lateral follicle cells (Fig. 5H-J). This difference was observed after early stage 9 and was Gurken dependent. The high endocytic activity in lateral follicle cells could set up a sharp boundary for the Gurken morphogen between the dorsal and ventral side of the egg chamber. This hypothesis is consistent with the quantification model (Goentoro et al., 2006), which predicted a steep reduction for the Gurken morphogen, from 63% to 22% at the lateral region. Thus, in response to early Gurken/Egfr activity, during stage 9 and later, once free Gurken binds to the Egfr in lateral follicle cells, the ligand-receptor complex may be internalized quickly as these lateral follicle cells may have a very active endocytic machinery. Because of a high activity of a degradation mechanism in the lateral follicle cells, only very low levels of Gurken would then be able to travel to the ventral region, leading to a low level of Egfr signaling in the ventral follicle cells, which would ensure the robustness of the Gurken gradient.

We thank Jean-Paul Vincent for the HRP plasmid, and Trudi Schüpbach for encouraging this project from the initial stage and for providing *gurken* genomic DNA, cDNA and fly stocks. We also thank Marcos González-Gaitán and Cheng-Ting Chien for providing fly stocks. Many thanks to the Microscope Facility at CGU for the excellent assistance with confocal microscopy. We are grateful to Trudi Schüpbach, Laura Nilson, Y-Henry Sun, Cheng-Ting Chien, Jui-Chou Hsu, Chien-Kuo Lee and Tzu-Chien V. Wang for critical reading of this manuscript. This work was supported by grants from the Chang-Gung Memorial Hospital (CMRPD140201-2 to L.P. and CMRPD340232 to W.L.), the National Science Council of Republic of China (NSC 95-2311-B-182-005 to L.P.) and the Ministry of Education, Taiwan, ROC (EMRPD170121 to L.P.).

**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/11/1923/DC1

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