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RESEARCH REPORT

Evolutionary changes in TGFα distribution underlie morphological diversity in eggshells from *Drosophila* species

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ABSTRACT

Drosophila eggshells display remarkable morphological diversity among species; however, the molecular origin of this structural diversification is mostly unknown. Here, we analyzed the dorsal ridge (DR), a lumen-like structure along the dorsal side of eggshells, from numerous *Drosophila* species. This structure varies in length and width across species, and is absent from *D. melanogaster* eggshells. We associated DR formation with distinct spatiotemporal changes in epidermal growth factor receptor (EGFR) activation, which acts as a key receptor in eggshell patterning. We show that changes in the distribution of the TGFα-like ligand Gurken (GRK), a crucial ligand for axis formation, underlies EGFR activation and DR formation in *D. willistoni*. Furthermore, we demonstrate that GRK from *D. willistoni* rescues a *grk*-null *D. melanogaster* fly and, remarkably, it is also sufficient to generate a DR-like structure on its eggshell.

KEY WORDS: Morphological novelty, EGFR activation, Eggshell, Gurken, Oogenesis

INTRODUCTION

Morphology is a highly diverse trait in nature; however, our understanding of the developmental and molecular mechanisms controlling the evolution of morphologies is still limited (Carroll, 2005, 2008). The *Drosophila* eggshell is the three-dimensional (3D) structure engulfing the developing embryo and displays different morphologies among species (Hinton, 1969; Kagesawa et al., 2008; Niepielko et al., 2011, 2012; Perrimon and Duffy, 1998). Given the high sensitivity of eggshell structures to changes in the levels of signaling, it provides an exceptional system to investigate how cell signaling regulates tissue morphology (Kagesawa et al., 2008; Niepielko et al., 2011; Ward and Berg, 2005). Numerous research groups have been focusing on the formation of respiratory dorsal appendages (DAs) (e.g. Boisclair Lachance et al., 2009; Dobens et al., 1997; Hinton, 1969; Marmion et al., 2013; Neuman-Silberberg and Schupbach, 1993; Osterfield et al., 2013; Peri and Roth, 2000; Sapir et al., 1998; Ward and Berg, 2005; Yakoby et al., 2008a). We investigated the formation of the lumen-like dorsal ridge (DR) along the dorsal-most side of the eggshell. The DR is absent from *D. melanogaster* eggshells but is found on Hawaiian *Drosophila* (Margaritis et al., 1983; Piano et al., 1997), *D. nebulosa*, *D. willistoni* (*Sophophora* subgenus) (Niepielko et al., 2014) and *D. cardini* eggshells (*Drosophila* subgenus) (Fig. 1A-D; supplementary material Fig. S1). Here, we show that changes in the distribution of the TGFα ligand Gurken (GRK) underlie DR formation. Remarkably, GRK from *D. willistoni* is sufficient to produce a DR on *D. melanogaster* eggshells.

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RESULTS AND DISCUSSION

DR morphologies are consistent with EGFR activation patterns

The DR is different among species and is absent from *D. melanogaster* eggshells. For example, the structure is wide, and reaches the posterior end in *D. nebulosa*, unlike the shorter structure in *D. willistoni* (Fig. 1B,C) (Niepielko et al., 2014), or the long and narrow structure in *D. cardini* (Fig. 1D). Analysis of the expression pattern of a family of chorion protein (Cp) genes in *D. melanogaster*, *D. willistoni*, and *D. nebulosa*, has revealed clustered expression patterns spanning the future DR domain with expression domains that are regulated by EGFR signaling (Niepielko et al., 2014).

Hypothesizing that the pattern of EGFR activation is different along the future DR domain of the various species, we stained egg chambers for diphosphorylated ERK (dpERK, encoded by the *rolled* gene – FlyBase), a downstream target of the EGFR activation cascade (Zartman et al., 2011). At stage 10, the dpERK pattern in *D. melanogaster* is restricted to the dorsal midline with a posterior extension of 49.14% (±1.3, s.e.m.) of the oocyte length (Fig. 1E) (Peri et al., 1999). Interestingly, in species that have DRs, the patterns of dpERK reflected the final shape and size of the DR morphologies (Fig. 1). For example, dpERK showed posterior extensions of 77.5% (±1.3), 96.1 (±1.1) and 94.7% (±1.1) of the oocyte lengths in *D. willistoni*, *D. nebulosa* and *D. cardini*, respectively (Fig. 1F-H). With the exception of the difference between *D. nebulosa* and *D. cardini*, the dpERK lengths were significantly different among species ($P<0.00001$). The full dynamics of EGFR activation in all four species are presented in supplementary material Figs S2 and S3.

GRK distribution is consistent with EGFR activation patterns

In *D. melanogaster*, the early activation of EGFR signaling is regulated by the oocyte-secreted GRK ligand, which is localized near the oocyte nucleus (Boisclair Lachance et al., 2009; Neuman-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1994; Zartman et al., 2009). The position of the nucleus in the oocyte sets the source of the GRK ligand, and therefore determines the activation gradient of EGFR in the overlying follicle cells (FCs) (Nilson and Schupbach, 1998; Van Buskirk and Schupbach, 1999). Focusing on GRK, we aimed to determine whether the localization of *grk* mRNA and/or GRK protein can account for different patterns of EGFR activations.

During *D. melanogaster* oogenesis, the *grk* mRNA is produced in the germline and localizes near the oocyte nucleus (Gonzalez-Reyes et al., 1995; Neuman-Silberberg and Schupbach, 1993; Queenan et al., 1997). The localized *grk* serves as a source of GRK protein that is translated and secreted to the perivitelline space, where it generates an EGFR activation gradient in the overlaying FCs

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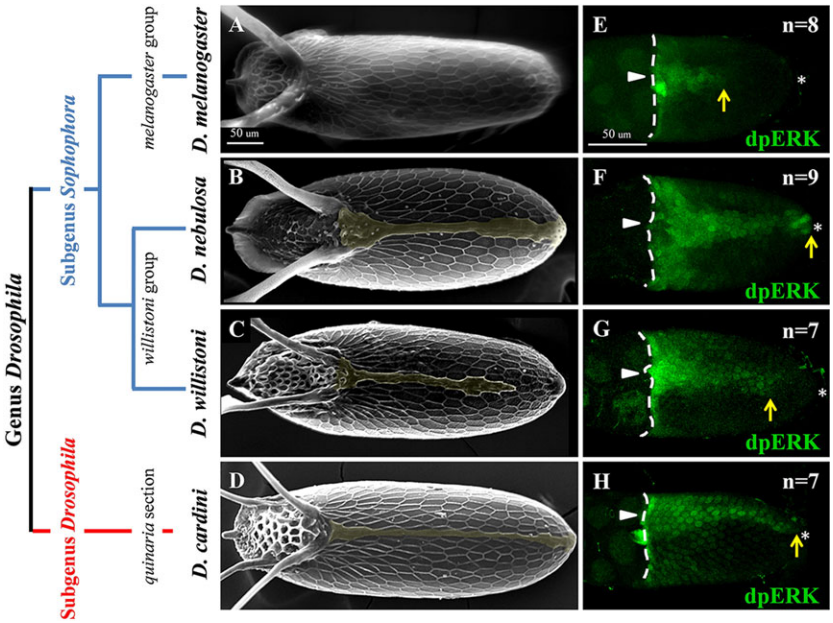

Fig. 1. DR morphologies are consistent with EGFR activation patterns. Phylogenetic tree with the corresponding subgenera *Sophophora* and *Drosophila*, and group or section. The lengths of the branches represent the phylogenetic arrangement of species, but not the evolutionary distance (Niepielko et al., 2011). (A-D) SEM images of eggshells. (A) *D. melanogaster* has no DR. (B-D) *D. nebulosa*, *D. willistoni* and *D. cardini* eggshells have a DR. The DR is artificially colored in yellow for clarity (uncolored eggshells are shown in supplementary material Fig. S1). All eggshells are dorsal views and anterior end is to the left. (E-H) Dorsal views of EGFR activation (dpERK, green). White arrowheads denote the dorsal midline. Dashed white lines denote the anterior FCs overlaying the oocyte. Yellow arrows denote the most posterior region of dpERK patterns, and the asterisks represent the most posterior region of the oocyte. Representative image out of *n* images examined.

(Goentoro et al., 2006a; Neuman-Silberberg and Schupbach, 1993; Thio et al., 2000). The mRNA of oocyte-secreted TGFα-like ligands in other animals, including *Tribolium* sp. (beetle) and *Gryllus* sp. (cricket), are not strictly localized near the oocyte nucleus (Lynch et al., 2010). Testing *grk* localization, we found that, like in *D. melanogaster*, *grk* localizes around the oocyte nucleus in all tested species (supplementary material Fig. S4). Thus, the localization of *grk* cannot account for the changes in EGFR activation.

Next, we aimed to determine whether the localization of GRK protein could account for the patterns of dpERK in species with and without a DR. The pattern of GRK in *D. melanogaster* is well characterized (Neuman-Silberberg and Schupbach, 1994; Van Buskirk and Schupbach, 1999). At late stage 9, the GRK protein localizes near the oocyte nucleus and has a posterior extension of 51% (±1.76, s.e.m.) of the oocyte length (Fig. 2A,A′,B; supplementary material Fig. S5). Using anti-GRK antibodies specific for *D. willistoni* and *D. cardini*, we found that, in addition to being localized around the oocyte nucleus, GRK had a posterior extension of 76% (±1.02) and 86% (±1.74) of the oocyte length, respectively (Fig. 2C-F). The lengths of GRK patterns among species were significantly different ($P<0.0001$). In summary, the patterns of dpERK, GRK and the shapes of the final DR structures are highly consistent.

GRK is necessary for DR formation

To associate GRK with DR formation, we knocked down *grk* in a *D. willistoni* using species-specific RNA interference (RNAi) constructs, and used *grk* knockdown in *D. melanogaster* as a positive control (Fig. 3A). This method has been previously used successfully to disrupt the TGFα-like ligand in the oocyte of the beetle, wasp and cricket (Lynch et al., 2010). We took advantage of the minimal heat shock promoter to drive the expression of *grk* RNAi constructs (see Materials and Methods for details). In *D. melanogaster*, we observed three types of DAs: severe (5%), fused (68%) and wild type (WT, 27%) (Fig. 3B-D). Over 70% of

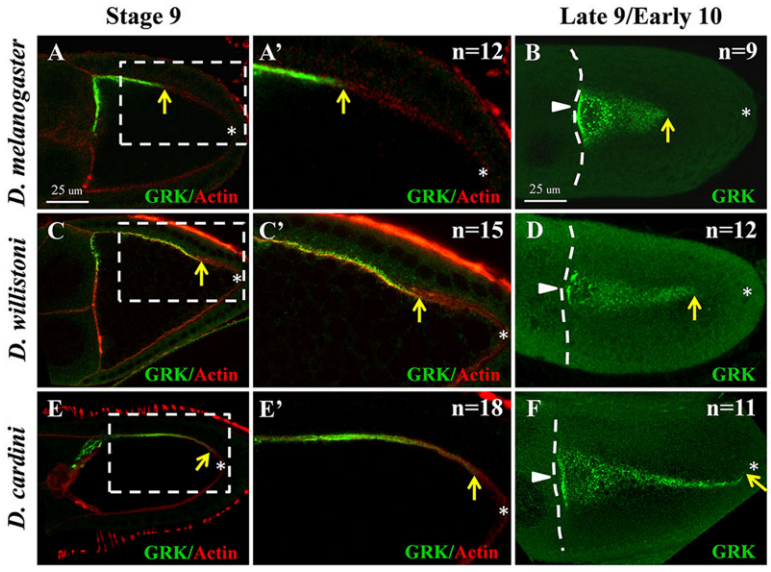

Fig. 2. Localization of GRK protein is different among species. Sagittal (A,C,E) and dorsal (B,D,F) views of egg chambers stained for Gurken protein (GRK, green) and actin (phalloidin, red). (A,B) *D. melanogaster*, (C,D) *D. willistoni* and (E,F) *D. cardini*. A′, C′ and E′ show magnified views of the area marked by a white dashed box in A, C and E, respectively. Dashed white lines denote the anterior FCs overlaying the oocyte. Yellow arrows denote the most posterior region of GRK patterns. Arrowheads denote the dorsal midline. Asterisks denote the posterior end of the oocyte. Representative image out of *n* images examined.

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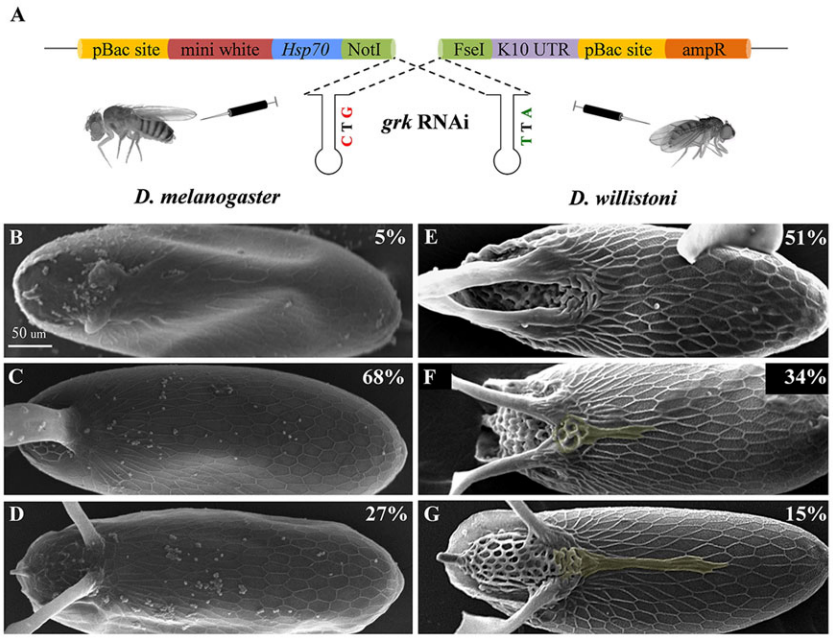

Fig. 3. Gurken RNAi disrupts eggshell morphologies. (A) A schematic representation of the UASpBacNPF injection vector (Holtzman et al., 2010) used to carry the *grk* RNAi constructs for *D. melanogaster* (left) and *D. willistoni* (right). The two nucleotide differences between the two *grk* RNAi constructs are denoted in red and green. Eggshell phenotypes of transgenic knockdown of *grk* in *D. melanogaster* (B-D) and *D. willistoni* (E-G). Eggshell phenotypes in *D. melanogaster*: (B) extreme, no dorsal appendages (DAs); (C) a single DA; and (D) WT-like DR. Eggshell phenotypes in *D. willistoni*: (E) extreme, no DR; (F) moderate, incomplete DR; and (G) WT-like DR. All eggshells are dorsal views with anterior to the left. The DR is artificially colored yellow. Percentages are the values for the indicated phenotype from of total counts of 194 and 134 eggshells of *D. melanogaster* and *D. willistoni*, respectively.

the heat shock-treated *grk* RNAi flies had some level of disrupted eggshells. The controls, heat-shocked wild-type flies and *grk* RNAi flies kept at 18°C, had mostly wild-type eggshells (supplementary material Table S1i). These phenotypes are consistent with eggshells with disrupted EGFR signaling (Neuman-Silberberg and Schupbach, 1993).

In heat shock-treated *grk* RNAi *D. willistoni*, we observed three types of eggshell morphologies. The DR was disrupted in 85% of the eggshells (Fig. 3E,F). In 51% of the eggshells, the DR was completely absent (Fig. 3E). In 34% of the eggshells, a few constricted cells near the base of the DAs were found, and there was reduced secretion of chorion material (Fig. 3F). A wild-type appearance was only observed in 15% of the eggshells (Fig. 3G). Of note, eggshells from heat shock-treated wild-type *D. willistoni* flies were also affected; however, the majority of eggshells had moderate and wild-type phenotypes (81%) (supplementary material Table S1ii). When *grk* RNAi flies were kept at 18°C, 98% of the eggshells were WT. The RNAi treatment successfully reduced the levels of GRK and dpERK (supplementary material Fig. S6), further supporting the hypothesis that GRK is necessary for DR formation. Interestingly, in most cases, the DAs of *grk* RNAi *D. willistoni* were still present (Fig. 3E-G), suggesting that DR formation is more sensitive to changes in the levels of EGFR activation.

D. willistoni grk rescues *D. melanogaster grk*-null flies and is sufficient to form a DR

The *grk* gene is rapidly evolving among *Drosophila* species and outside of Diptera (Lynch et al., 2010). The moderate sequence identity among *grk* genes is restricted to a few domains, including the signal peptide and EGF domain (Peri et al., 1999); thus, it is uncertain whether *grk* from one species can rescue another. Previously, germline cells were exchanged between *D. virilis* and *D. melanogaster*; however, the progeny contained *grk* from both species (Nakamura et al., 2007). Here, we inserted a *grk* locus from *D. willistoni* into *D. melanogaster* (Fig. 4A). We found that *D. willistoni* GRK (*w*GRK) RNA and protein were correctly localized to the dorsal anterior in the transgenic *D. melanogaster* flies (supplementary material Fig. S7).

To determine the function of the transgenic *w*GRK in eggshell morphology, we compared eggshell morphologies in *D. melanogaster* flies with that from flies with two additional copies of *D. melanogaster* GRK (*m*GRK) (Neuman-Silberberg and Schupbach, 1993) or *w*GRK. As expected, the gap between the two DAs is wider with the addition of extra copies of GRK, with the most substantial increase caused by the additional two copies of *m*GRK (supplementary material Fig. S8). Associated with the addition of two copies of *w*GRK, we found a clear increase in the area posterior to the base of the DAs (supplementary material Fig. S8C), suggesting that *w*GRK can mediate the formation of a domain posterior to the base of the DAs.

To test the particular contribution of *w*GRK, we crossed the transgenic *w*GRK fly into a *grk*-null fly, obtaining progeny with one or two copies of *w*GRK. Four distinct eggshell phenotypes were observed (Fig. 4B). Most eggshells, 69% and 78% from one and two copies of *w*GRK, respectively, were designated as Class I (WT) or Class II (WT with an enlarged area posterior to the base of the DAs); a phenotype similar to the that in wild-type *D. melanogaster* with two additional copies of *w*GRK (supplementary material Fig. S8C). As far as we know, this is the first time it has been demonstrated that *grk* from a different species can rescue *grk*-null *D. melanogaster*. Remarkably, a DR-like morphology was found in 10% and 1% of the eggshells from one and two copies of *w*GRK, respectively (Fig. 4B, Class III; supplementary material Fig. S9). The low penetrance of eggshells with a DR is consistent with the few egg chambers found to have an elongated distribution of *w*GRK along the future DR domain (supplementary material Fig. S7D,D′). Morphologically, this DR has a similar lumen to *D. nebulosa* and *D. willistoni* (Fig. 4C-E). In 13% and 18% of the eggshells from *D. melanogaster* with one and two copies of *w*GRK, respectively, we noticed a single or fused DA (Fig. 4B, Class IV). In 8% and 3% of the eggshells from *D. melanogaster* with one and two copies of *w*GRK, respectively, we noticed collapsed unmountable eggs. We suggest that the DR is regulated by lower levels of signaling provided by GRK in the future DR domain, whereas the operculum domain is regulated by high levels of EGFR signaling, mediated by the GRK originating from the nucleus. In this case, two copies of *w*GRK increased the type II eggshells in a position posterior to the base of the dorsal appendages, which is in the same location that one copy

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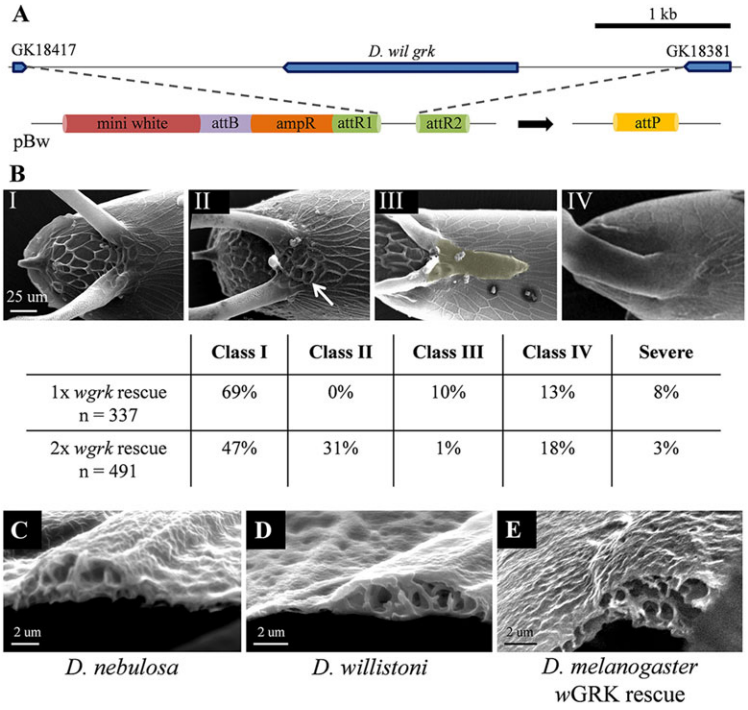

	Class I	Class II	Class III	Class IV	Severe
1x *wgrk* rescue n = 337	69%	0%	10%	13%	8%
2x *wgrk* rescue n = 491	47%	31%	1%	18%	3%

D. nebulosa *D. willistoni* *D. melanogaster* *w*GRK rescue

Fig. 4. *w*GRK rescues a *grk*-null fly of *D. melanogaster*. (A) The entire *grk* locus of *D. willistoni* (*w*GRK) was inserted into a modified pBPGUw (pBw) and injected into *D. melanogaster* at position 68A4 of the attP2 fly. Transgenic flies were crossed to a *grk*-null background. (B) Eggshells of transgenic flies were scored as follows: Class I, WT; Class II, having an enlarged area in a position posterior to the base of the DAs, denoted by a white arrow; Class III, having a DR-like structure (artificially colored yellow); Class IV, having a single or fused DA; Severe, collapsed unmountable eggs (not shown in the eggshell images). The table summarizes the phenotypic distributions of eggshells from flies with one copy (1× *wgrk* rescue) and two copies (2× *wgrk* rescue) of *w*GRK. (C-E) Cross-section of the DR domain from eggshells of (C) *D. nebulosa* flies, (D) *D. willistoni* flies and (E) transgenic *w*GRK *D. melanogaster* flies that are null for *grk*. All images are dorsal views and anterior is to the left.

generated a DR (Fig. 4B). Similar results were obtained when the *w*GRK was inserted on the X chromosome of *D. melanogaster*; thus, the integration site cannot account for DR formation.

The EGF domain of *m*GRK and *w*GRK has ~56% identity and contains six conserved cysteine residues that are necessary to form the EGF domain (supplementary material Fig. S10). The gap between cysteine residues 3 and 4 has been suggested to affect EGFR activation by other ligands, including Vein and Spitz (Schnepp et al., 1998). A comparison between the two EGF domains reveals a one amino acid difference in the gap between cysteine residues 3 and 4 (supplementary material Fig. S10), which might lead to a stronger signal in the FCs overlaying the oocyte nucleus and might account for the wider gap between the two DAs that is mediated by *m*GRK (supplementary material Fig. S8). Although *w*GRK can induce DR formation in *D. melanogaster*, we could not find a protein domain that was responsible for the difference between *m*GRK and *w*GRK. In the future, a rigorous domain swapping between *m*GRK and *w*GRK is required to determine what domain in *w*GRK mediates DR formation.

Several mechanisms have been shown to regulate EGFR signaling, including negative regulators, extracellular matrix proteins and co-receptors (Boisclair Lachance et al., 2009; Mao and Freeman, 2009; Wang et al., 2008; Zartman et al., 2009). It will be important to determine whether trans-acting elements can provide further local regulation of EGFR signaling to increase the robustness of DR formation through anchoring GRK along its entire path without affecting the dorsal-ventral axis of the fly. Regardless of the specific domain, in recent years, most research associated with the evolution of morphologies has focused on changes in cis regulatory modules as an underlying mechanism (Carroll, 2005, 2008). Here, we show that different distributions of GRK proteins, and not *grk* RNA patterns, can account for the evolution of DR formation.

MATERIALS AND METHODS

Flies

Flies strains used were: *D. melanogaster* (OreR), *D. nebulosa*, *D. cardini*, (UC San Diego *Drosophila* Stock Center), *D. willistoni* (a gift from David Stern, Janelia Research Campus, Ashburn, VA, USA), *D. willistoni* pBac-Blue eye (Holtzman et al., 2010), w^- *D. melanogaster grk* null [2b]*b, grk* null [2E12]*b* (gifts from Trudi Schüpbach, Howard Hughes Medical Institute, Princeton University, Princeton, NJ, USA), X7;28.20 (Neuman-Silberberg and Schupbach, 1993), and w^- *D. melanogaster*. *w*GRK was inserted on the third chromosome at position 68A4 of the attP2 fly (Genetic Services) and into the X chromosome of the ZH-2A fly (Rainbow Transgenics). All flies were maintained on standard cornmeal food.

Gene cloning

cDNA construction and PCR cloning were performed as described previously (Goentoro et al., 2006b; Niepielko et al., 2011; Yakoby et al., 2008a). Primers for partial *grk* are found in supplementary material Table S2. Genomic DNA was isolated from *D. willistoni* (Vienna *Drosophila* Rnai Center/VDRC protocol). The *D. willistoni grk* locus was amplified using the Qiagen long-range PCR kit and protocol with primers found in supplementary material Table S2. *D. willistoni grk* locus was TOPO-cloned into pCR8 plasmid (Invitrogen). The pCR8 vector containing the *D. willistoni grk* locus was Gateway-cloned using an LR reaction between a modified pBPGUw (pBw) (Pfeiffer et al., 2008) (Addgene, 17575) vector and pCR8-*will-grk* with Invitrogen LR II clonase. Modification of pBw included the exclusion of the Gal4, terminator and promoter regions using *Fse*I and *Xba*I, followed by ligation of the annealed primers 5′-CCCTAGCCCTGCAGGCT-3′ and 5′-CTAGAGCCTGGAG-GGCTAGGGCCGG-3′ into the two restriction sites. pBw-*will-grk* was inserted into *D. melanogaster* at position 68A4 of the attP2 fly (Genetic Services).

dpERK staining, immunoassay, *in situ* hybridization and microscopy

Staining for dpERK was performed as previously described (Zartman et al., 2009), using rabbit anti-dpERK antibody (Cell Signaling) at 1:100. The oocyte nucleus was stained with mouse anti-Half Pint antibody (1:100) (Van Buskirk and Schupbach, 2002) and DAPI (1:10,000). *D. melanogaster* mouse anti-Gurken antibody (1D12, DSHB, IA) was used 1:10. Polyclonal mouse anti-Gurken antibodies for *D. willistoni* and *D. cardini* were provided by PrimmBiotech (see supplementary material Fig. S11). Pre-absorbed antibodies were used at 1:100 as described previously (Yakoby et al., 2008b). Actin was stained using phalloidin (1:100) (Life Technologies). *In situ* hybridization was performed as previously described (Wang et al.,

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2006; Yakoby et al., 2008a). Egg chambers were imaged using a Leica SP8 confocal microscope (except for Fig. 2, where images were taken using a Leica SP5 confocal microscope; Imaging Core Facility, Princeton University, NJ, USA). Images were processed with ImageJ (NIH). Scanning electron microscopy (SEM) images were obtained as described previously (Niepielko et al., 2014). The DR was artificially colored using Photoshop (Adobe). The *n* values are the number of additional images that represent each pattern.

RNAi constructs, injection and heat shock

Short hairpin RNAi against *grk* were designed as described previously (Haley et al., 2008). Oligonucleotides for the top and bottom strands of *D. willistoni* and *D. melanogaster* RNAi strands were designed for the *Fse*I and *Not*I sites (supplementary material Table S2). Annealed strands were ligated into UASpBacNPF vector digested with *Not*I and *Fse*I (Bio-Labs) (Holtzman et al., 2010) and electroporated into DH5α *E. coli* bacteria. Vectors containing RNAi constructs were recovered using the Qiagen midi prep kit and sequenced (GENEWIZ). Plasmids were injected into w^- *D. melanogaster* (Genetic Services) and pBac-Blue-eyed *D. willistoni* (Rainbow Transgenics) as previously described (Holtzman et al., 2010). Gurken RNAi flies were heat-shocked for 1 h at 37°C three times a day [vectors have a heat shock promoter (Holtzman et al., 2010)].

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.G.N. and N.Y. designed the research, analyzed the data, and wrote the paper. M.G.N. performed the research.

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Supplementary material

Supplementary material available online at
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References

Boisclair Lachance, J.-F., Fregoso Lomas, M., Eleiche, A., Bouchard Kerr, P. and Nilson, L. A. (2009). Graded Egfr activity patterns the Drosophila eggshell independently of autocrine feedback. *Development* **136**, 2893-2902.

Carroll, S. B. (2005). Evolution at two levels: on genes and form. *PLoS Biol.* **3**, e245.

Carroll, S. B. (2008). Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. *Cell* **134**, 25-36.

Dobens, L. L., Hsu, T., Twombly, V., Gelbart, W. M., Raftery, L. A. and Kafatos, F. C. (1997). The Drosophila bunched gene is a homologue of the growth factor stimulated mammalian TSC-22 sequence and is required during oogenesis. *Mech. Dev.* **65**, 197-208.

Goentoro, L. A., Reeves, G. T., Kowal, C. P., Martinelli, L., Schüpbach, T. and Shvartsman, S. Y. (2006a). Quantifying the Gurken morphogen gradient in Drosophila oogenesis. *Dev. Cell* **11**, 263-272.

Goentoro, L. A., Yakoby, N., Goodhouse, J., Schüpbach, T. and Shvartsman, S. Y. (2006b). Quantitative analysis of the GAL4/UAS system in Drosophila oogenesis. *Genesis* **44**, 66-74.

González-Reyes, A., Elliott, H. and St Johnston, D. (1995). Polarization of both major body axes in Drosophila by gurken-torpedo signalling. *Nature* **375**, 654-658.

Haley, B., Hendrix, D. A., Trang, V. and Levine, M. (2008). A simplified miRNA-based gene silencing method for *Drosophila melanogaster*. *Dev. Biol.* **15**, 282-290.

Hinton, H. E. (1969). Respiratory systems of insect egg shells. *Annu. Rev. Entomol.* **14**, 343-368.

Holtzman, S., Miller, D., Eisman, R. C., Kuwayama, H., Niimi, T. and Kaufman, T. C. (2010). Transgenic tools for members of the genus Drosophila with sequenced genomes. *Fly (Austin)* **4**, 349-362.

Kagesawa, T., Nakamura, Y., Nishikawa, M., Akiyama, Y., Kajiwara, M. and Matsuno, K. (2008). Distinct activation patterns of EGF receptor signaling in the homoplastic evolution of eggshell morphology in genus Drosophila. *Mech. Dev.* **125**, 1020-1032.

Lynch, J. A., Peel, A. D., Drechsler, A., Averof, M. and Roth, S. (2010). EGF signaling and the origin of axial polarity among the insects. *Curr. Biol.* **20**, 1042-1047.

Mao, Y. and Freeman, M. (2009). Fasciclin 2, the Drosophila orthologue of neural cell-adhesion molecule, inhibits EGF receptor signalling. *Development* **136**, 473-481.

Margaritis, L. H., Dellas, K., Kalantzi, M. C. and Kambysellis, M. P. (1983). The eggshell of Hawaiian *Drosophila*: structural and biochemical studies in *D. grimshawi* and comparison to *D. melanogaster*. *Wilhelm Roux's Arch. Dev. Biol.* **192**, 303-316.

Marmion, R. A., Jevtic, M., Springhorn, A., Pyrowolakis, G. and Yakoby, N. (2013). The Drosophila BMPRII, wishful thinking, is required for eggshell patterning. *Dev. Biol.* **375**, 45-53.

Nakamura, Y., Kagesawa, T., Nishikawa, M., Hayashi, Y., Kobayashi, S., Niimi, T. and Matsuno, K. (2007). Soma-dependent modulations contribute to divergence of rhomboid expression during evolution of Drosophila eggshell morphology. *Development* **134**, 1529-1537.

Neuman-Silberberg, F. S. and Schupbach, T. (1993). The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. *Cell* **75**, 165-174.

Neuman-Silberberg, F. S. and Schupbach, T. (1994). Dorsoventral axis formation in Drosophila depends on the correct dosage of the gene gurken. *Development* **120**, 2457-2463.

Niepielko, M. G., Hernáiz-Hernández, Y. and Yakoby, N. (2011). BMP signaling dynamics in the follicle cells of multiple Drosophila species. *Dev. Biol.* **354**, 151-159.

Niepielko, M. G., Ip, K., Kanodia, J. S., Lun, D. S. and Yakoby, N. (2012). The evolution of BMP signaling in *Drosophila* oogenesis: a receptor-based mechanism. *Biophys. J.* **102**, 1722-1730.

Niepielko, M. G., Marmion, R. A., Kim, K., Luor, D., Ray, C. and Yakoby, N. (2014). Chorion patterning: a window into gene regulation and Drosophila species' relatedness. *Mol. Biol. Evol.* **31**, 154-164.

Nilson, L. A. and Schüpbach, T. (1998). EGF receptor signaling in Drosophila oogenesis. *Curr. Top. Dev. Biol.* **44**, 203-243.

Osterfield, M., Du, X., Schüpbach, T., Wieschaus, E. and Shvartsman, S. Y. (2013). Three-dimensional epithelial morphogenesis in the developing Drosophila egg. *Dev. Cell* **24**, 400-410.

Peri, F. and Roth, S. (2000). Combined activities of Gurken and decapentaplegic specify dorsal chorion structures of the Drosophila egg. *Development* **127**, 841-850.

Peri, F., Bökel, C. and Roth, S. (1999). Local Gurken signaling and dynamic MAPK activation during Drosophila oogenesis. *Mech. Dev.* **81**, 75-88.

Perrimon, N. and Duffy, J. B. (1998). Developmental biology: sending all the right signals. *Nature* **396**, 18-19.

Pfeiffer, B. D., Jenett, A., Hammonds, A. S., Ngo, T.-T. B., Misra, S., Murphy, C., Scully, A., Carlson, J. W., Wan, K. H., Laverty, T. R. et al. (2008). Tools for neuroanatomy and neurogenetics in Drosophila. *Proc. Natl. Acad. Sci. USA* **105**, 9715-9720.

Piano, F., Craddock, E. M. and Kambysellis, M. P. (1997). Phylogeny of the island populations of the Hawaiian Drosophila grimshawi complex: evidence from combined data. *Mol. Phylogenet. Evol.* **7**, 173-184.

Queenan, A. M., Ghabrial, A. and Schupbach, T. (1997). Ectopic activation of torpedo/Egfr, a Drosophila receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. *Development* **124**, 3871-3880.

Sapir, A., Schweitzer, R. and Shilo, B. Z. (1998). Sequential activation of the EGF receptor pathway during Drosophila oogenesis establishes the dorsoventral axis. *Development* **125**, 191-200.

Schnepp, B., Donaldson, T., Grumbling, G., Ostrowski, S., Schweitzer, R., Shilo, B.-Z. and Simcox, A. (1998). EGF domain swap converts a drosophila EGF receptor activator into an inhibitor. *Genes Dev.* **12**, 908-913.

Thio, G. L., Ray, R. P., Barcelo, G. and Schüpbach, T. (2000). Localization of gurken RNA in Drosophila oogenesis requires elements in the 5′ and 3′ regions of the transcript. *Dev. Biol.* **221**, 435-446.

Van Buskirk, C. and Schüpbach, T. (1999). Versatility in signalling: multiple responses to EGF receptor activation during Drosophila oogenesis. *Trends Cell Biol.* **9**, 1-4.

Van Buskirk, C. and Schüpbach, T. (2002). Half pint regulates alternative splice site selection in Drosophila. *Dev. Cell* **2**, 343-353.

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Wang, X., Bo, J., Bridges, T., Dugan, K. D., Pan, T.-c., Chodosh, L. A. and Montell, D. J. (2006). Analysis of cell migration using whole-genome expression profiling of migratory cells in the Drosophila ovary. *Dev. Cell* **10**, 483-495.

Wang, P.-Y., Chang, W.-L. and Pai, L.-M. (2008). Smiling Gurken gradient: an expansion of the Gurken gradient. *Fly (Austin)* **2**, 118-120.

Ward, E. J. and Berg, C. A. (2005). Juxtaposition between two cell types is necessary for dorsal appendage tube formation. *Mech. Dev.* **122**, 241-255.

Yakoby, N., Bristow, C. A., Gong, D., Schafer, X., Lembong, J., Zartman, J. J., Halfon, M. S., Schüpbach, T. and Shvartsman, S. Y. (2008a). A combinatorial code for pattern formation in Drosophila oogenesis. *Dev. Cell* **15**, 725-737.

Yakoby, N., Lembong, J., Schupbach, T. and Shvartsman, S. Y. (2008b). Drosophila eggshell is patterned by sequential action of feedforward and feedback loops. *Development* **135**, 343-351.

Zartman, J. J., Kanodia, J. S., Cheung, L. S. and Shvartsman, S. Y. (2009). Feedback control of the EGFR signaling gradient: superposition of domain-splitting events in Drosophila oogenesis. *Development* **136**, 2903-2911.

Zartman, J. J., Cheung, L. S., Niepielko, M. G., Bonini, C., Haley, B., Yakoby, N. and Shvartsman, S. Y. (2011). Pattern formation by a moving morphogen source. *Phys. Biol.* **8**, 045003.