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.

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 germ line 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 overlying FCs.
(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′; 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 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.

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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 (wGRK) 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 wGRK in eggshell morphology, we compared eggshell morphologies in D. melanogaster flies with that from flies with two additional copies of D. melanogaster GRK (mGRK) (Neuman-Silberberg and Schupbach, 1993) or wGRK. 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 mGRK (supplementary material Fig. S8). Associated with the addition of two copies of wGRK, we found a clear increase in the area posterior to the base of the DAs (supplementary material Fig. S8C), suggesting that wGRK can mediate the formation of a domain posterior to the base of the DAs.

To test the particular contribution of wGRK, we crossed the transgenic wGRK fly into a grk-null fly, obtaining progeny with one or two copies of wGRK. Four distinct eggshell phenotypes were observed (Fig. 4B). Most eggshells, 69% and 78% from one and two copies of wGRK, 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 that in wild-type D. melanogaster with two additional copies of wGRK (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 wGRK, 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 morphology was found in 10% and 1% of the eggshells from one and two copies of wGRK, respectively (Fig. 4B, Class IV; supplementary material Fig. S8C). The low penetrance of eggshells with a DR is consistent with the few egg chambers found to have an elongated morphology.
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 comparison between the two EGF domains reveals a one amino acid 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 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 Fsel and Xbal, followed by ligation of the annealed primers 5′-CCCTAGGCTGACCGCT-3′ and 5′-CTAGAGCTTGAG-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, DSIB, 1A) 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, 2008).
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 (Niepelko 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 grrk 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 Fsel and Norl sites (supplemental material Table S2). Annealed strands were ligated into UASPbacNPF vector digested with Norl and Fsel (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 (Rainbow Transgenics) as previously described (Holtzman et al., 2010).

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

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