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Source: Zoological Science, 33(6) : 583-591

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zs160105

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ZOOLOGICAL SCIENCE **33**: 583–591 (2016) © 2016 Zoological Society of Japan

An RNAi Screen for Genes Involved in Nanoscale Protrusion Formation on Corneal Lens in *Drosophila melanogaster*

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The "moth-eye" structure, which is observed on the surface of corneal lens in several insects, supports anti-reflective and self-cleaning functions due to nanoscale protrusions known as corneal nipples. Although the morphology and function of the "moth-eye" structure, are relatively well studied, the mechanism of protrusion formation from cell-secreted substances is unknown. In *Drosophila melanogaster*, a compound eye consists of approximately 800 facets, the surface of which is formed by the corneal lens with nanoscale protrusions. In the present study, we sought to identify genes involved in "moth-eye" structure, formation in order to elucidate the developmental mechanism of the protrusions in *Drosophila*. We re-examined the aberrant patterns in classical glossy-eye mutants by scanning electron microscope and classified the aberrant patterns into groups. Next, we screened genes encoding putative structural cuticular proteins and genes involved in cuticular formation using eye specific RNAi silencing methods combined with the Gal4/UAS expression system. We identified 12 of 100 candidate genes, such as cuticular proteins family genes (*Cuticular protein 23B* and *Cuticular protein 49Ah*), cuticle secretion-related genes (*Syntaxin 1A* and *Sec61 β subunit*), ecdysone signaling and biosynthesis-related genes (*Ecdysone receptor*, *Blimp-1*, and *shroud*), and genes involved in cell polarity/cell architecture (*Actin 5C*, *shotgun*, *armadillo*, *discs large1*, and *coracle*). Although some of the genes we identified may affect corneal protrusion formation indirectly through general patterning defects in eye formation, these initial findings have encouraged us to more systematically explore the precise mechanisms underlying the formation of nanoscale protrusions in *Drosophila*.

Key words: *Drosophila melanogaster*, cuticle, corneal protrusion, "moth-eye" structure, nanostructure, RNAi screening, biomimetics

INTRODUCTION

Many insects possess a compound eye consisting of hundreds or thousands of ommatidial arrays. Each ommatidium comprises a set of photoreceptor cells, and its surface is covered with the corneal lens, through which light reaches the photoreceptive area, i.e., the rhabdom. Bernhard and Miller (1962) discovered that the outer surfaces of ommatidial lens are covered with an array of cuticular protrusions termed corneal nipples. The cone-shaped protrusions are 200–300 nm in height, and spaced in nearly hexagonal arrays. The nanoscale protrusion serves an anti-reflective function (Bernhard et al., 1963). Moreover, a recent study revealed that the self-cleaning property occurs due to the reduction of adhesive forces between contaminating materials and biological lens surfaces (Peisker and Gorb, 2010).

The corneal lens is one form of insect exoskeleton, the cuticle, and thus, the basic structure of lens is the same as that of the cuticle (Perry, 1968). The cuticle comprises a multi-layered structure, consisting of envelope, epicuticle, and procuticle (for review, see Locke, 2001). The outermost envelope functions to prevent water loss and is composed of proteins and lipids. Beneath it, the epicuticle functions to provide the stiffness of the cuticle and is composed of cross-linked proteins. The innermost procuticle confers elasticity to the cuticle and consists of chitin fiber-protein lattices. The formation of the multi-layered structure in the lens proceeds from the outermost to innermost layer (Gemne, 1971). Although the morphology and function of corneal protrusions have been studied, the molecular mechanism of nanoscale protrusion formation remains unclear.

In *D. melanogaster*, the nanoscale protrusions are also present on the lens surface of the ommatidia and ocelli (Stark et al., 1989). An ommatidium contains a set of eight photoreceptor cells, four cone cells, and two primary pigment cells, which is surrounded by three secondary pigment cells, three tertiary pigment cells, and three bristle cells. It is known that the cone cells and primary pigment cells secrete most components of the corneal lens, and secondary pig-

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Supplemental material for this article is available online.
doi:10.2108/zs160105

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ment cells also secrete corneal materials at the boundary between ommatidia (for review, see Charlton-Perkins and Cook, 2010). It has been reported that classical glossy eye fly mutants, such as *glass* (*gl*) (Stark and Carlson, 1991), *lozenge* (*lz*) (Stark and Carlson, 2000), and *sparkling* (*spa*) (Oster and Crang, 1972), possess aberrant "moth-eye" surfaces. Recently, atomic force microscopy (AFM) analysis of glossy eyes overexpressing Wingless showed a dramatic loss of corneal nano-structures (Kryuchkov et al., 2011). Thus, corneal nanoscale protrusions should be formed under genetic regulation. Given the availability of many powerful genetic tools, *D. melanogaster* thus is an excellent insect model for investigating the developmental mechanisms that give rise to corneal protrusions.

In the present study, we sought to identify genes involved in the formation of corneal nanoscale protrusions as a means to begin to elucidate the mechanism in *Drosophila*. A better understanding of this phenomenon would allow us to more fully appreciate the intricate landscape of the ordered nanoscale structures using the genetically amenable fruit fly, to contribute to the field of self-organization of secreted substances from cells in developmental biology, and to adapt novel bioengineering methods to the creation of bioinspired applications, such as anti-reflective and self-cleaning functions, inspired by the "moth-eye" structure.

MATERIALS AND METHODS

Fly stocks and crossings for screening

All flies were reared on yeast-glucose-cornmeal-agar medium at 25°C. *Canton-Special* (*CS*) flies were used as wild-type controls. Four classical glossy-eye mutants, *Glazed* (*Gla:* a dominant allele of *wingless*), lz^3, gl^1, and spa^{pol} were obtained from the Bloomington stock center and Kyoto Stock Center. In our screen to identify genes involved in the corneal nanoscale protrusion formation, the RNAi-mediated knockdown of a gene was induced using the Gal4/UAS system (Brand and Perrimon, 1993). UAS-RNAi transgenic fly lines were obtained from the Vienna Drosophila Resource Center (VDRC) and the National Institute of Genetics (NIG) RNAi stocks. The information is summarized in Supplementary Tables S1 and S2 online. These RNAi lines were crossed with an eye-specific Gal4 driver, *GMR-Gal4*, which expresses Gal4 under the control of *glass multiple reporter* (*GMR*) promoter elements (from Bloomington stock center), and the corneal protrusion patterns of F1 progeny were examined using a scanning electron microscope (SEM). When the F1 progeny showed lethality at the pharate adult stage, pharate adults were dissected out of the puparium, the pupal cuticle was removed, and then the corneal protrusion patterns were examined, if possible.

Scanning electron microscopy

Adult female flies were fixed for 30 min in 3.7% formaldehyde in PBS-0.006% TritonX-100, washed once for 15 min in PBS-0.3% TritonX-100, twice for 15 min in distilled water, and serially dehydrated with 25%, 50%, 75%, and 90% ethanol for more than 15 min and with 100% ethanol overnight. After dehydration, ethanol was replaced with 50% tert-butyl alcohol for 15 min and then twice with 100% tert-butyl alcohol for 30 min. Then, the sample was frozen at 4°C for 15 min. Finally, flies were further dehydrated by freeze-drying using a freeze dryer (VFD-21S, Vacuum Device Inc). Specimens were subjected to gold-coating by an ion coater (Neo coater MP-19010 NCTR, JEOL), and the corneal lens was observed using a SEM (TM3000 Miniscope, Hitachi).

RESULTS AND DISCUSSION

Corneal protrusion patterns in *Drosophila*

The *Drosophila* compound eye is composed of approximately 800-unit facets or ommatidia that arranged in a precise hexagonal array (Fig. 1A). Hair-like structures called interommatidial bristles are present between the ommatidial lenses (Fig. 1A'). Cone-shaped nanoscale protrusions exist on the surface of the corneal lens (Fig. 1A'), and the protrusions are arranged in an approximately regular hexagonal close-packed pattern (Fig. 1A"). We also observed nanoscale protrusions with number of five or seven nearest neighbors in wild type flies. According to our measurement by SEM, the distance adjacent to the tip of the protrusions was about 250 nm in wild type flies, which corroborates previous AFM studies (Kryuchkov et al., 2011).

Aberrant corneal protrusion patterns in classical glossy-eye mutants

We observed the corneal surface patterns in four classical viable mutants, *Gla*, lz^3, gl^1, and spa^{pol}, with glossy eyes by SEM, and typical features of aberrant patterns of corneal protrusions in the mutants were classified (Fig. 1B"–E"). The ommatidial lens of *Gla* mutants had a flat surface (Fig. 1B), and the nanoscale protrusions were either not evident or diminished in these lens surfaces (no-protrusion type; Fig. 1B' and B"). The genes *lz*, *spa*, and *gl* are known to encode transcription factors crucial for pre-patterning photoreceptor precursors (Daga et al., 1996) and photoreceptor cell development (Fu and Noll, 1997; Moses et al., 1989). Loss-of-function mutant alleles lz^3, gl^1, and spa^{pol} displayed small ommatidial sizes, irregular alignment, and defects of the dome shape in the corneal lens (Fig. 1C–E and C'–E'). We observed the grainy cuticle structure in lz^3 ommatidial surface (grainy type; Fig. 1C"). In gl^1 and spa^{pol} mutants, we observed elongated protrusions, which suggested that some nanoscale protrusions were fused in the ommatidial surface (fusion type; Fig. 1D" and E"). There were also maze-like structures in the spa^{pol} mutant (maze type; Fig. 1E"). The maze type may arise from many fused protrusions connected over a wide range. In addition, nanoscale protrusions disappeared partially in lens of the lz^3, gl^1, and spa^{pol} mutants (Fig. 1C', D' and E'). In addition to the four phenotypes categorized in the classical glossy-eye mutants, we have identified another type of aberrant protrusion whose size is larger than that of wild type (enlarged type; Fig. 1F–F", see below). Thus, we recognize five aberrant protrusion patterns in total.

Screening of the candidate genes functioning in corneal protrusion formation

To elucidate the molecular and cellular mechanisms of the nanoscale protrusion formation, we carried out RNAi screening of genes in which their knockdowns induced aberration of corneal protrusion formation. Specifically, we induced RNAi in candidate genes with the eye specific *GMR-Gal4* driver and observed corneal protrusion patterns in these flies. We focused on two types of candidate genes: cuticular protein (CPR) family genes, and genes involved in cuticular formation. There are 102 putative CPR family genes, which are characterized by a conserved region

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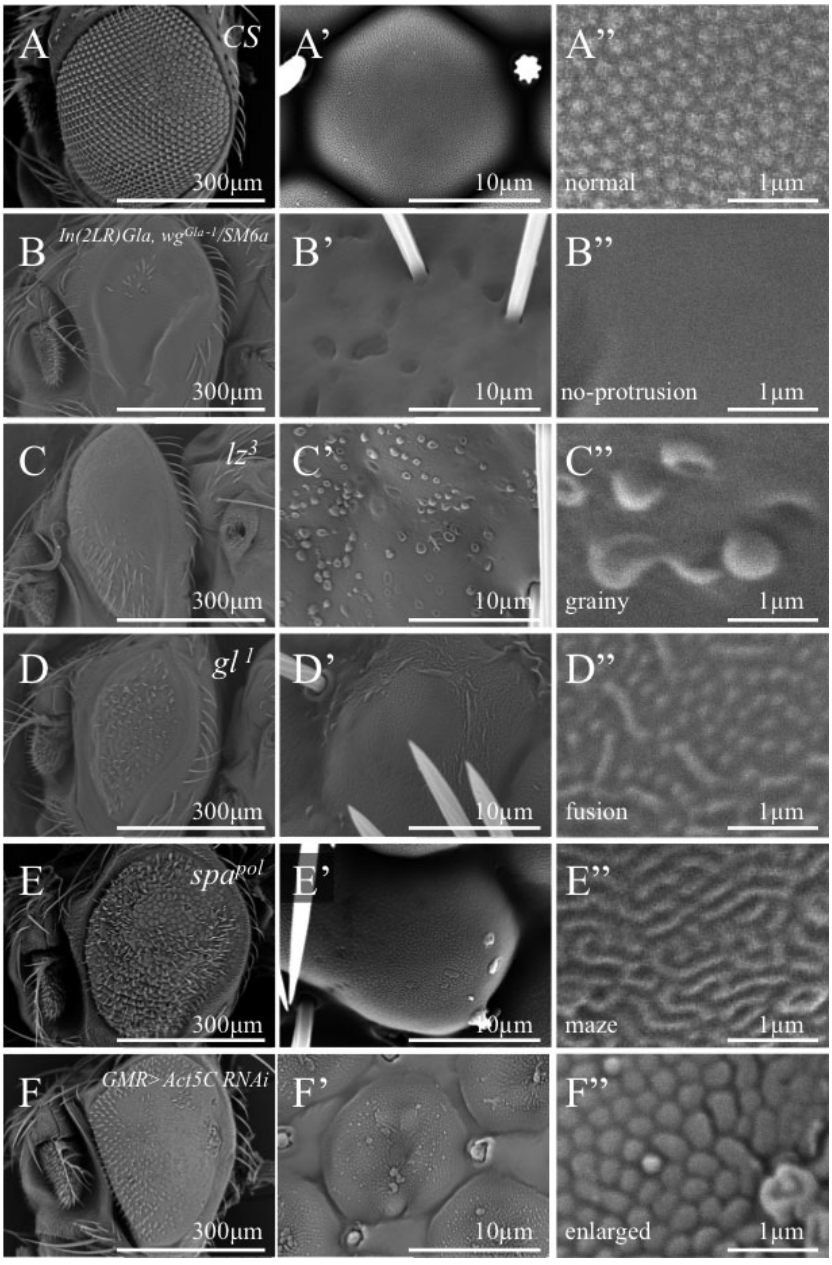


Fig. 1. Scanning electron micrograph of six types of corneal protrusion patterns in wild-type flies, classical glossy-eye mutants and *Act5C* knockdown flies. **(A–A")** wild-type flies *CS* (normal type). **(B–B")** $y^1\ w^{67c23}$*; In(2LR)Gla,* $wg^{Gla\text{-}1}$*/SM6a* (no-protrusion type). **(C–C")** lz^3 (grainy type). **(D–D")** gl^1 (fusion type). **(E–E")** spa^{pol} (maze type). **(F–F")** *w; GMR-Gal4/UAS-Act5C RNAi* (*HMS02487*) (enlarged type). Scale bars: 300 μm **(A–F)**, 10 μm **(A'–F')**, 1 μm **(A"–F")**.

known as the chitin-binding Rebers and Riddiford Consensus (R&R Consensus) (Cornman, 2009). The functions of most CPR family genes remain unknown. In this study, we screened 68 CPR family genes (107 RNAi lines) (Supplementary Table S1) and identified two genes, *Cpr23B* and *Cpr49Ah*, which were crucial for proper corneal protrusion formation (Table 1). We then investigated the influence of RNAi knockdown of genes that affect the cuticle development of the body surface at the embryonic, larval, and adult phases. These genes belong to five categories: chitin assembly, sclerotization and melanization, secretion, ecdysone signaling, and cell polarity (Schwarz and Moussian, 2007). We screened 32 genes from these five categories and identified 10 genes crucial for corneal protrusion formation (Supplementary Table S2). Overall, we identified 12 genes (Table 1) out of 100 candidate genes. The effects of RNAi knockdown and impact on corneal protrusion formation for each category are detailed in the following sections.

Cuticular protein (CPR) family genes

RNAi-mediated knockdown of *Cpr23B* and *Cpr49Ah* that resulted in the reduction of the activity of two genes induced aberrant protrusion formation. In these knockdown flies, we frequently observed glossy and rough compound eyes with fused ommatidia (Fig. 2A and B). Corneal protrusions of *Cpr23B* knockdown flies were enlarged (Fig. 2A' white arrowhead), while the *Cpr49Ah* knockdown flies had maze type and grainy type protrusions (Fig. 2B' black arrow and open white arrowhead, respectively). Additionally the protrusions disappeared partially at the margin of the corneal lens in *Cpr23B* and *Cpr49Ah* knockdown flies (Fig. 2A' and B' asterisks).

The roles of *Cpr23B* and *Cpr49Ah* genes were not well known with regards to cuticle formation. One recent study reported that a mutation of *Cpr49Ah* resulted in thin and misaligned wing hair phenotypes (Adler et al., 2013). Hence, the *Cpr49Ah* is also required for proper cuticle formation of the lens, as well as the wing hair.

Genes involved in cuticular formation

Chitin assembly

The procuticle is composed of a protein-chitin matrix that confers elasticity to the cuticle. Chitin's composition of β 1,4-linked N-acetylglucosamine (GlcNAc) keenly contributes to the arthropod exoskeleton (for review, see Muthukrishnan et al., 2012). GlcNAc is converted into activated monomers by the uridine diphosphate (UDP)-GlcNAc pyrophosphorylase encoded by the *mummy* (*mmy*) gene (Araújo et al., 2005), and chitin fibers are synthesized by Chitin Synthase-1 encoded by the *krotzkopf verkehrt* (*kkv*) gene (Moussian et al., 2005a). The chitin fibers protrude into the extracellular space, where the chitin–protein complex is organized. Apical membrane-anchored extracellular proteins, encoded by the *retroactive* (*rtv*) and *knickkopf* (*knk*) genes, play an important role in organizing and orientating chitin fibers (Moussian et al., 2005b; Moussian et al., 2006). Another membrane-associated protein encoded by *piopio* (*pio*) has been shown to be required for attachment of the procuticle to the apical epidermal surface (Bökel et al., 2005). In addition to mem-

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Table 1. Effects of RNAi knockdown of the candidate genes on protrusion formation.

	Gene Name	Molecular function	Protrusion type
CPR family genes	***Cpr23B***	Insect cuticle protein (Chitin-binding type R&R consensus)	no-protrusion (at the margin), enlarged
	Cpr49Ah	Insect cuticle protein (Chitin-binding type R&R consensus)	no-protrusion (at the margin), grainy, maze
Secretion	***Syx1A***	SNARE binding	no-protrusion (at the margin), grainy, fusion, enlarged
	Sec61β	Protein transporter activity	no-protrusion (at the margin), enlarged
Ecdyson signaling	***EcR***	Ecdysone signalling targets	no-protrusion (at the margin), grainy, maze
	Blimp-1	Transcriptional repressor activity	no-protrusion
	sro	Glucose/ribitol dehydrogenase	grainy, enlarged
Cell polarity/cell architecture	***Actin5C***	Structure constituent of cytoskeleton	grainy, fusion, enlarged
	shg	Cell adhesion molecule binding (AJ)	no-protrusion (at the margin), grainy, fusion, enlarged
	arm	Cell adhesion molecule binding (AJ)	grainy, fusion, enlarged
	dlg1	Structural molecule activity (SJ)	no-protrusion (at the margin)
	cora	Cytoskeletal protein binding (SJ)	no-protrusion (at the center)

AJ; adherens junction, SJ; septate junction.

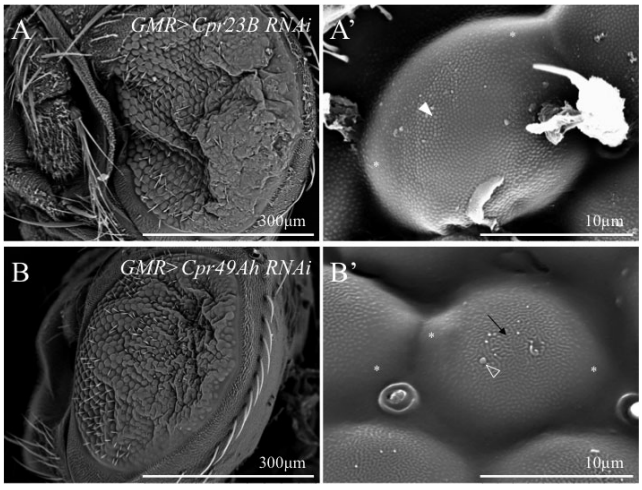


Fig. 2. Scanning electron micrograph of corneal protrusion patterns in *Cpr* knockdown flies. **(A, A')** *w/UAS-Cpr23B RNAi 2973R-1; GMR-Gal4/+*. **(B, B')** *w; GMR-Gal4/+; UAS-Cpr49Ah RNAi 8515R-1/+*. White arrowhead in **(A')**: enlarged type. Asterisks in **(A')** and **(B')**: the protrusions disappeared partially at the margin (no-protrusion type). Open white arrowhead in **(B')**: grainy type. Black arrow in **(B')**: maze type. Scale bars: 300 μm **(A, B)**, 10 μm **(A', B')**.

brane-associated factors, apical extracellular matrix chitin deacetylase proteins, encoded by the *vermiform* (*verm*) and *serpentine* (*serp*) genes, function in the organization and orientation of chitin fibers (Luschnig et al., 2006). Moreover, Schlaff (encoded by *slf*) is one of the unidentified factors affecting cuticle differentiation and structure (Nüsslein-Volhard et al., 1984).

We examined the effect of RNAi-mediated knockdown of these eight genes on corneal protrusion formation. We did not detect any aberrations of the protrusion pattern in flies with knockdowns of *mmy*, *knk*, *rtv*, *serp*, *verm*, *slf*, and *pio* genes (Supplementary Table S2). The effect of *kkv* knockdown could not be examined, because *kkv* knockdown was lethal at the pharate adult stage, and the surface of their eyes was often ruptured and melanized. Mutant embryos for *kkv*, which lose chitin in the cuticle, have been shown in previous studies to display abnormally soft cuticles with poor mechanical integrity (Moussian et al., 2005a). Likewise, loss of corneal chitin should cause similar defects in lens formation. Thus, in our screening, most of chitin biosynthesis factors do not directly affect the formation of protrusion pattern. Chitin defects induced in the procuticle layer may not affect the envelope and epicuticle layers where corneal protrusion formation proceeds. However, we note that our RNAi knockdown system may be insufficiently powerful to induce corneal protrusion defects.

Sclerotization and melanization

Cuticle differentiation requires the process of hardening (sclerotization) and tanning (melanization), which are induced by some catecholamine metabolites with related proteins and enzymes. Tyrosine is converted into dopa by the enzyme tyrosine hydroxylase encoded by the *pale* (*ple*) gene (Neckameyer and White, 1993), and dopa is subsequently converted into dopamine by dopa decarboxylase, encoded by the *Dopa decarboxylase* (*Ddc*) gene (Bishop and Wright, 1987), or into N-β-alanyl dopamine (NBAD) by β-alanyl dopamine synthase encoded by the *ebony* (*e*) gene (Richardt et al., 2003). These catecholamines which are synthesized in epidermal cells are released into cuticle layers as precursors for sclerotization and melanization, in which phenol oxidase catalyzes them into their respective quinone derivatives. Quinones such as NBAD-quinone bind covalently to cuticlar proteins that function in cuticle sclerotization. Dopa- and dopamine-quinones are converted to melanogenic substrates through the enzymatic activity of Ebony encoded by the *ebony* (*e*) gene, aspartate decarboxylase encoded by the *black* (*b*) gene (Phillips et al., 2005), NBAD hydrolase encoded by the *tan* (*t*) gene (True et al., 2005), and carboxypeptidase D (CPD) encoded by the *silver* (*svr*) gene (Settle et al., 1995). Additionally, the *yellow* (*y*) gene is known to be involved in cuticle melanization (Riedel et al., 2011).

We examined the effect of knockdown in the *pale*, *Ddc*, *e*, *b*, *t*, *svr*, and *y* on corneal protrusion formation (12 RNAi lines). No aberrant protrusion formations were observed in these knockdown flies (Supplementary Table S2). This result is plausible because no pigmentation occurs in lens cuticle, and sclerotization would proceed after corneal protrusion formation. Usually, after eclosion, secretion of the bursicon hormone causes sclerotization, whereas corneal protrusion formation occurs during the middle of the pupal stage in the fruit fly (Yamahama and Kimura, unpublished data) as well as the moth, *Manduca sexta* (Gemne, 1971). Thus, the factors for cuticular sclerotization and melaniza-

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tion are not involved in corneal protrusion formation.

Secretion

Recently, secretion mechanisms in cuticle differentiation have been investigated (Valcárcel et al., 1999; Abrams and Andrew, 2005; Moussian et al., 2007). We examined the effect of knockdown on corneal protrusion formation in seven cuticle secretion-related genes: *Syntaxin 1A* (*Syx1A*) which codes for a product that interacts with the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) (Schulze et al., 1995); *Sec61 β subunit* (*Sec61β*) which encodes a subunit of Sec61 endoplasmic reticulum (ER) translocon protein (Valcárcel et al., 1999); *Coat Protein γ* (*γCOP)* gene that encodes a subunit of the Coat Protein Complex (COP) I coatomer complex, which is trafficked primarily from the early Golgi to the endoplasmic reticulum (ER) (Abrams and Andrew, 2005); and *Sar1*, *Sec31*, *haunted* (*hau: Sec23*) and *ghost* (*gho*) genes which collectively encode component proteins of the COP II complex that mediates protein transport from the ER to Golgi (Norum et al., 2010). Out of the seven genes examined, RNAi knockdown of two genes, *Syx1A* and *Sec61β* induced severe defects in corneal protrusion formation (Supplementary Table S2). Ommatidia in *Syx1A* knockdown flies were frequently fused and decreased in size, especially at the posterior region of the eye (Fig. 3A). Protrusions on the corneal lens were enlarged at the center and disappeared at the margin (Fig. 3A' white arrowhead and asterisks) and frequently fused to form a line (Fig. 3A' white arrow). Moreover, there were grainy structures (Fig. 3A' open white arrowheads). In *Sec61β* knockdown ommatidia, protrusions disappeared at the margin of the lens (Fig. 3B and B' asterisks) and were unequally distributed at the center of the lens where protrusions appeared to be irregular in size (Fig. 3B' white arrowhead). Thus, defects of protein trafficking affect corneal protrusion formation, a finding that is further supported by the fact that the protein epicuticle layer, in which protrusions are formed in the corneal lens, is absent in *Drosophila Sec61β* mutant embryos (Valcárcel et al., 1999). The corneal protrusion pattern in *Sec31* knockdown ommatidia appeared to be normal (data not shown). *Sar1, γCOP*, *hau*, and *gho* knockdown flies could not be examined because they were lethal during the pharate adult stage and possessed fragile lens (data not shown) like the *kkv* knockdown flies. The membrane vesicle trafficking via COP I and COP II is a major secretory route responsible for full cuticle differentiation (Abrams and Andrew, 2005; Norum et al., 2010). To investigate the effects of those lethal genes on corneal protrusion formation, we will consider using a weak Gal4-driver line to induce RNAi or hypomorphic mutations.

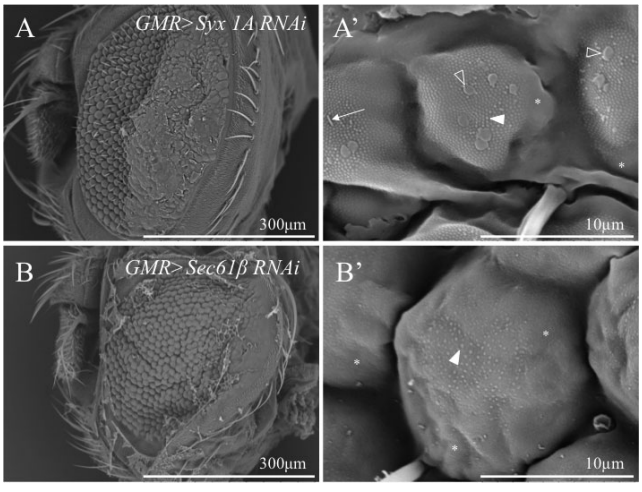


Fig. 3. Scanning electron micrograph of corneal protrusion patterns in flies with RNAi knockdown of genes involved in cuticle secretion. **(A, A')** *w; GMR-Gal4/UAS-Syx1A RNAi R-3*. **(B, B')** *w; GMR-Gal4/+; UAS-Sec61β RNAi 8785/+*. Open white arrowheads in **(A')**: grainy type. White arrowheads in **(A')** and **(B')**: enlarged type. Asterisks in **(A')** and **(B')**: the protrusions disappeared partially at the margin (no-protrusion type). White arrow in **(A')**: fusion type. Scale bars: 300 μm **(A, B)**, 10 μm **(A', B')**.

Ecdysone signaling

In *Drosophila*, ecdysteroids, including ecdysone and its derivative 20-hydroxyecdysone (20E), are important for cuticle formation (Doctor et al., 1985). Response to ecdysteroids are mediated by a functional Ecdysone receptor (EcR)/Ultraspiracle (Usp) heterodimer (Thomas et al., 1993; Yao et al., 1993). Moreover, the 20E is known to directly induce the *B lymphocyte-induced maturation protein 1* (*Blimp-1*) gene which regulates the timing of the ecdysone-induced developmental pathway (Agawa et al., 2007).

We assessed the effect of gene knockdown in two ecdysone signaling genes, *EcR* and *Blimp-1* (Supplemetary Table S2). We observed abnormalities at the center region of the compound eye upon the knockdown of *EcR* and *Blimp-1* (Fig. 4A and B). In *EcR* knockdown corneal lens, the fused protrusions merged into a maze at the center region (Fig. 4A' black arrow) and the periphery contained a partial loss of protrusions (Fig. 4A' asterisks). Moreover, huge grainy protrusions surrounded at the outermost region (Fig. 4A' open white arrowheads). We observed the fusion type and grainy type as a weak phenotype of the *EcR* knockdown at the center region of the lens (data not shown). *Blimp-1* knockdown lens also showed no protrusions and a rough surface (Fig. 4B' asterisk). It has been suggested that there is a relationship between ecdysone signaling and secretion of the cuticle. It was reported that ecdysone induces Golgi formation by driving the transcription of a number of genes coding for factors of the ER and Golgi, such as the COP II component *Sec23* in the imaginal discs (Dunne et al., 2002; Kondylis et al., 2001). These findings suggest that secretion and membrane trafficking may be a fundamental response to the hormone pulse.

The genes involved in ecdysone biosynthesis are known as Halloween-group genes, mutations of which cause loss of ecdysone during embryogenesis, resulting in cuticle differentiation defects (Chávez et al., 2000). We examined the gene-silencing effect of one of the Halloween-group genes, shroud (*sro*), which encodes a short-chain dehydrogenase/reductase (Niwa et al., 2010). We often observed fused ommatidia in *sro* knockdown flies (Fig. 4C). The corneal protrusions were irregular in size (Fig. 4C' white arrowhead) and of the grainy type (Fig. 4C' open white arrowhead). In the pupal stage, ecdysone and its derivative 20E are secreted from the gland cells into the hemolymph and act on the epidermis to regulate cuticle formation. Since *sro* gene-

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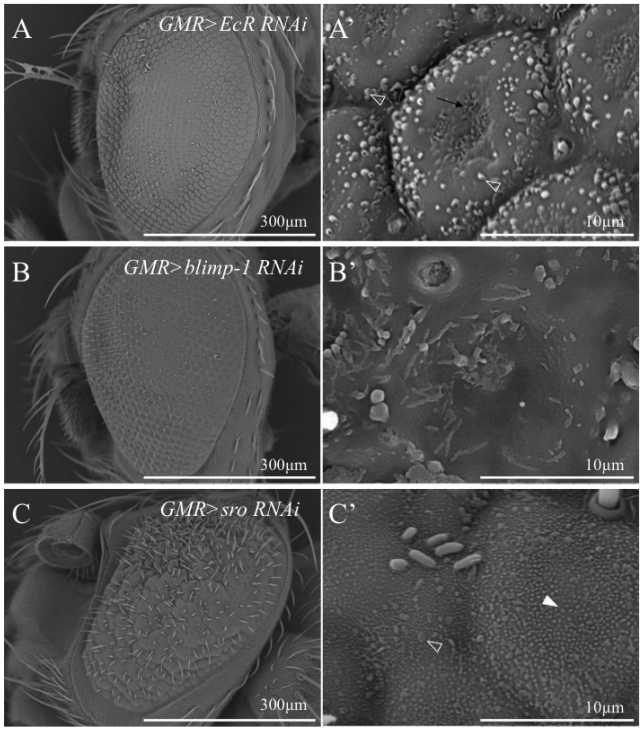


Fig. 4. Scanning electron micrograph of corneal protrusion patterns in flies with RNAi knockdown of genes involved in ecdysone signaling and biosynthesis. **(A, A')** *w; GMR-Gal4/UAS-EcR RNAi 9327*. **(B, B')** *w; GMR-Gal4/+; UAS-blimp-1 RNAi R-2/+*. **(C, C')** *w; GMR-Gal4/+; UAS-sro RNAi 16386/+*. Open white arrowheads and black arrow in **(A')**: grainy type and maze type, respectively. Asterisks in **(A', B')**: the protrusions disappeared partially at the margin (no-protrusion type). Open white arrowhead in **(C')**: grainy type. White arrowhead in **(C')**: enlarged type. Scale bars: 300 μm **(A–C)**, 10 μm **(A'–C')**.

knockdown is induced only within the ommatidial cell, this effect on corneal protrusions may not be caused by ecdysone directly. Sro is a member of the Short-chain dehydrogenase/reductase enzymes, which play roles in lipid, amino acid, carbohydrate, cofactor, and hormone metabolism in a wide range of cells and tissues (Kavanagh et al., 2008). Therefore, the *sro* gene might have some unknown function in corneal lens cuticle formation besides ecdysteroid biosynthesis.

Cell polarity/cell architecture

Cell polarity provides information for the correct localization of certain factors to the apical plasma membrane for secretion of cuticle material to the extracellular matrix. One of the planar cell polarity genes *Drosophila* E-cadherin (DE-cadherin), encoded by the *shotgun* (*shg*) gene, is required for proper embryonic cuticle development (Tepass et al., 1996). DE-cadherin forms a complex with α-catenin encoded by the *α-cat* gene, β-catenin encoded by the *armadillo* (*arm*) gene, and adherens junction (AJ) protein p120, which is collectively called the cadherin/catenin complex. This cadherin/catenin complex binds to filamentous actin, and is essential in proper cell adhesion (Pai et al., 1996). The *crumbs* (*crb*) gene encodes a large transmembrane protein required for maintenance of apico-basal cell polarity and AJ in embryonic epithelia (Tepass et al., 1990; Tepass and Knust, 1990). Moreover, insects have a semi-permeable barrier that called the septate junctions (SJ) at basal region of the AJ. Two genes, *discs large 1* (*dlg1*) and *coracle* (*cora*), are well known as SJ component genes (Woods and Bryant, 1993; Fehon et al., 1994). These genes are involved in cell polarity and serve as factors that maintain the cellular architecture.

We investigated the effect in these six genes on corneal protrusion formation: *shg*, *arm*, *crb*, *dlg1*, *cora*, and cytoskeletal actin gene (*Act5C*) (Supplementary Table S2). Five of the six genes silenced resulted in abnormal protrusion formation. We observed fused ommatidia in *Act5C*, *shg*, and *arm* knockdown flies (Fig. 5A–C). In particular, these flies exhibited enlarged protrusions (Fig. 5A'–C' white arrowheads) and occasionally had fused protrusions and grainy structures (Fig. 5A'–C' white arrows and open white arrowheads). In addition, in *shg* knockdown flies, protrusions partially disappeared at the lens surface margin (Fig. 5B' asterisks). The protrusion pattern in *crb* knockdown ommatidia, in contrast, appeared normal (data not shown). Interestingly, common effects of protrusion enlargement were induced by aberration of the cadherin/catenin complex. This implies that cell polarity or cell architecture regulated by intercellular adhesion and actin-based cytoskeleton controls the size of protrusions during its formation.

The *dlg1* knockdown flies possessed small compound eye sizes and defects of the dome shape in the ommatidial corneal lens (Fig. 5D). The protrusions disappeared partially at the margin in the ommatidia of *dlg1* knockdown flies as well (Fig. 5D' asterisks). Alternatively, *cora* knockdown eyes, the ommatidial alignment of which appeared to be normal, showed loss of protrusions at the center of the lens (Fig. 5E and E' asterisk). Although it is interesting that *dlg1* and *cora* knockdowns affect the reciprocal region, the cause of these phenotypes remains unclear.

We have shown that corneal protrusion formation is under the genetic control of various genes, and that these genes can be modulated to impact the dynamics of protrusion formation. However, it is important to consider that protrusion defects may be mediated by more general defects in eye patterning prior to protrusion formation. The genes identified here may affect corneal protrusion formation indirectly through general patterning defects in eye formation. Certainly, some genes, such as *arm*, *dlg* and *cora*, are known to be involved in eye patterning (Ahmed et al., 1998; Beronja et al., 2005; Legent et al., 2012; Lamb et al., 1998). Assessments of whether and how the respective genes are expressed in the lens-secreting cells at the time of protrusion formation would help to address whether the effects are direct or indirect. Additionally, we note two technical limitations of the RNAi knockdown approached used in this study. We did not control for the effectiveness of the RNAi constructs used to down-regulate the respective genes. Examining for the expression levels of the respective genes would be helpful to confirm the function of the gene in protrusion formation. In addition, we cannot rule out the possibility that some of the RNAi phenotypes were caused by off-target effects.

Various corneal protrusion patterns are observed in

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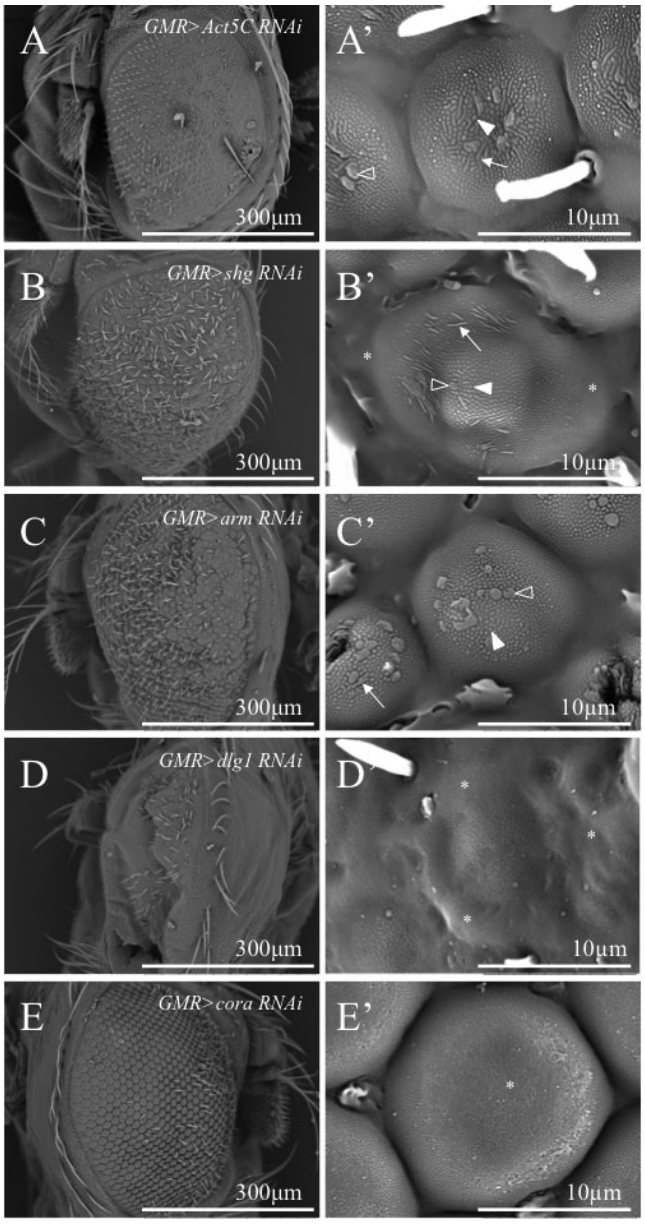


Fig. 5. Scanning electron micrograph of corneal protrusion patterns in flies with RNAi knockdown of genes involved in cell polarity/cell architecture. **(A, A')** *w; GMR-Gal4/CyO; UAS-Act5C RNAi (HMS02487)/TM6,Tb*. **(B, B')** *w; GMR-Gal4/UAS-shg RNAi R-3*. **(C, C')** *w; GMR-Gal4/UAS-arm RNAi R-1*. **(D, D')** *w; GMR-Gal4/UAS-dlg1 RNAi R-2*. **(E, E')** *w; GMR-Gal4/UAS-cora RNAi 9787*. White arrows, open white arrowheads and white arrowheads in **(A', B', C')**: fusion type, grainy type and enlarged type, respectively. Asterisks in **(B', D')** (at the margin) and E'(at the center): no-protrusion type. Scale bars: 300 μm **(A–E)**, 10 μm **(A'–E')**.

nature (Sukontason et al., 2008). Moreover, Blagodatski et al. (2015) provides a comprehensive analysis of the corneal protrusion pattern in 23 insect orders, some of which were similar to patterns resulting from gene knockdowns in our study, e.g., irregular protrusions of various sizes, strands merging into a maze, and observations of maze-like structures. These findings imply that evolutionary diversification of insect corneal protrusion patterns results from species-specific modification of gene regulation involved in corneal protrusion formation.

In corneal protrusion formation, Gemne (1971) proposed that the corneal protrusions originate from secretion by the regularly spaced microvilli of the cone lens cells by observing protrusion formation using transmission electron microscopy (TEM) in *Manduca*. Blagodatski et al. (2015) reported a rich diversity of insect corneal protrusion patterns among which transitions appeared, sometimes within the same lens, and suggested that formation on the tip of microvilli is not satisfactory to account for nanoscale protrusions. They proposed an alternative mathematical model based on the Turing reaction-diffusion mechanism, and were able to simulate various protrusion patterns found in nature. They hypothesized that the Turing reaction-diffusion mechanism for nanoscale protrusion pattern formation is mediated by organic components of the lens, possessing different diffusion properties and mutually influencing each other's abundance, polymerization, and aggregation. Although the molecular identity of the components or morphogens patterning corneal protrusions remains unknown, our studies may provide some insights into the components. For instance, knockdown of two CPR genes may provide potential insight about the materials needed for lens formation based on our phenotypes observed in our RNAi screening. Hence, these CPR genes might encode one of the components related to the Turing reaction-diffusion processes. In addition, we found that various corneal protrusion phenotypes are present on identical lens surfaces in RNAi knockdown flies. The expression level of Gal4 in the *GMR-Gal4* line was not uniform among lens-producing cells at the pupal protrusion formation stage, if membrane-bound GFP was driven (data not shown). Thus, mixed phenotypes on one lens may result from differences in the effects of RNAi-mediated knockdown in different lens-producing cells.

Interestingly, gene knockdown of cellular actin also influenced corneal protrusion formation in our screening. The microvillus core is composed of actin (Gemne, 1971), and the change of corneal protrusion patterns might therefore have been caused by defective microvilli. Even if the Turing mechanism does act in the formation of nanoscale protrusions as suggested by Blagodatski et al. (2015), microvilli may nonetheless act as the first scaffolds for this reaction. To test this hypothesis, we must examine the formative process of various protrusion patterns chronologically in mutants which were identified in this study.

"Moth-eye" structure-like corneal protrusions, which serve anti-reflective, self-cleaning, and/or water-repellent functions, has emerged at the forefront of nature-inspired biomimetic technology. While morphology and function of "moth-eye" structure are relatively studied as discussed earlier (Bernhard and Miller, 1962; Bernhard et al., 1963), the mechanism of the formation is still elusive. However, our initial attempts to identify the factors involved in the nanoscale protrusion formation on the corneal lens using the model insect, *D. melanogaster*, have encouraged us to further examine the precise mechanisms regulating nanoscale protrusion formations. Elucidation of the mechanism will inspire us to develop "moth-eye"-inspired nanostructured products.

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ACKNOWLEDGMENTS

We thank Kyoto Stock Center, NIG-FLY stock center, Bloomington Stock Centers and Vienna Drosophila RNAi Center for numerous *Drosophila* strains; H. Fudouji, S. Yoshioka, D. Ishii, M. Shimomura and Y. Uozu for valuable discussion; and A. Izumi for technical assistance. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan to K-I. K., H. K and T. H. (No. 24120004).

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(Received June 4, 2016 / Accepted August 2, 2016)

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