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Genetic link between *β-sarcoglycan* and the Egfr signaling pathway

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Abstract

Sarcoglycans are a multimeric, integral membrane protein complex that is part of the dystrophin glycoprotein complex. Previous findings suggest that the dystrophin glycoprotein complex plays roles not only in maintaining the mechanical structure of the cell membrane but also in signal transduction. To evaluate the functions of sarcoglycans, we here took advantage of *Drosophila*, which is useful for screening genetic interactions. Morphological aberrancy was observed in the adult compound eyes of *Drosophila β-sarcolgycan* (*dscgβ*) knockdown flies. We also detected genetic interactions between *dscgβ* and Egfr related genes, such as *rhomboid-1*, *rhomboid-3*, and *mirror*. Furthermore two extra cell types with strong expression of Rhomboid were found in the ommatidia of *dscgβ* knockdown pupal retina. These cells exhibited phosphorylation of ERKA, suggesting that Egfr signaling is activated via Rhomboid. Through these in vivo analyses, we conclude that *dscgβ* negatively regulates the Egfr signaling pathway.

Keywords: β-Sarcoglycan; Egfr; *Drosophila*; Rhomboid; Retina; βPS Integrin

The dystrophin glycoprotein complex (DGC) is a group of integral and membrane-associated proteins, which connect the extracellular matrix with the cytoskeleton [1]. Included are dystrophin, the dystroglycan complex, the sarcoglycan complex, sarcospan, syntrophins, and dystrobrevins [1]. While mutations in *dystrophin* are responsible for Duchenne-type muscular dystrophy and Becker-type muscular dystrophy, molecular changes in α-, *β*-, γ-, and *δ-sarcoglycan* cause autosomal recessive limb girdle muscular dystrophy (LGMD), type2D, type2E, type2C, and type2F, respectively. [2]

The sarcoglycan complex, composed of a combination of four single transmembrane sarcoglycans, plays a crucial role in the DGC [3]. One function is to stabilize the DGC itself in the cell membrane via direct interactions with dystroglycan, thus providing a strong mechanical linkage between the extracellular matrix and the intracellular actin cytoskeleton [3]. Many studies on diverse interacting proteins within the DGC and the features of each component have suggested that the DGC has a signaling role in addition to structural functions. For example, α-sarcoglycan has an ATP-binding site in the extracellular region, and α-sarcoglycan-transfected HEK293 cells exhibit a significant increase in ATP-hydrolyzing activity that is abolished by anti-α-sarcoglycan antibodies, suggesting the existence of ecto-ATPase activity [4]. Previous studies have shown that anti-dystrophin or anti-α-sarcoglycan co-precipitates integrin α5β1 and other focal adhesion-associated proteins, such as vinculin, talin, paxillin, and FAK, as well as the DGC, in cultured L6 rat myocytes [5]. Conversely, anti-β1-integrin antibodies co-precipitate the components of the DGC [5]. Immunostaining data for adult human normal skeletal muscle indicate co-localization of sarcoglycans and integrins at costameres [6,7], and severe reduction of β1D-integrin and filamin2 in adult human skeletal muscle has been documented for an LGMD type2D patient [8]. Furthermore, the phosphorylation state of sarcoglycan changes in response to mechanical perturbation and cell attachment [5,9]. These observations suggest the presence of bidirectional signaling between sarcoglycans and integrins[5,8]. Filamin 2, muscle specific filamin, has been identified as another signaling molecule that can interact

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with γ- and δ-sarcoglycans via its C-terminal region, using the yeast two-hybrid method [10]. This interaction can be inhibited by cleavage of filamin2 by calpain3, that is responsible for LGMD type2A [11]. Although various proteins interacting with DGC have been discovered and speculations have been made regarding related signaling pathways, little is known about definitive and initial upstream signals. In addition, while many recent studies have uncovered diverse functions of dystroglycan or dystrophin, fundamental roles for multifunctional sarcoglycans remain to be determined.

Recent elucidation of human and fly genomic sequences has revealed *Drosophila melanogaster* to possess fewer redundant orthologues of most human DGC components, with the exception of sarcospan, where three have been described (dScgα, dScgβ, and dScgδ, corresponding to α-/ε-, β-, γ-/δ-/ζ-sarcoglycan in human). The lower redundancy seen in *Drosophila* facilitates analysis of protein functions [12] and therefore *Drosophila* was utilized as an animal model in the present study.

In the mouse retina, β-, δ-, γ-, and ε-sarcoglycans are localized predominantly in the outer and the inner limiting membranes, probably in the Muller cells and also in the ganglion cell axons where the expression of dystrophins has never been reported. In Mdx mice that lack dystrophin, no significant change in expression and distribution of the sarcoglycan components in adult mouse retina was observed [13]. These observations suggest that expression and subcellular localization of the Sarcoglycan complex are at least partially independent of dystrophins. Consistently, in *Drosophila* eye imaginal discs, dScgβ was not completely colocalized with those of *Drosophila* dystroglycan and Syntrophin-2 (to be published elsewhere), suggesting that dScgβ has functions independent of other DGC components.

Transgenic flies carrying UAS-IR (inverted repeated)-*dscgβ* were first generated to investigate the effect of the knockdown of *dscgβ* in the eye. We found a rough eye phenotype accompanied by defects of ommatidial rotation and production of the ectopic cells in retina. In addition, Rhomboid could be shown to be strongly expressed not only in these ectopic cells but also in photoreceptor cells, causing activation of ERK via Egfr signaling. Furthermore, half-reduction of the *rhomboid* gene dose strongly suppressed the rough eye phenotype induced by the knockdown of *dscgβ*. These results indicate genetic link between dscgβ and Egfr signaling pathway.

Materials and methods

Fly stocks. Fly stocks were maintained at 25 °C on standard food containing 0.7% agar, 5% glucose, and 7% dry yeast. Canton S was used as the wild-type.

Alleles of the following genes were obtained from the Bloomington *Drosophila* stock center : $mirror^{SaiD3}$, ru^{1}, rho^{ve-1}, rho^{7M43}, and rho^{4A69}. Enhancer trap lines carrying the lacZ markers X63 (*rhomboid*), ro156 (*rough*), and AE127 (*seven-up*) were obtained from Y. Hiromi. *hspFlp; + ;tub1 > FRT cd2 FRT > GAL4,UAS-GFP/TM3(Sb)* was a kind gift from A. Plessis. Establishment of lines carrying GMR-GAL4 was as described earlier [14,15]. Act5C-GAL4 was also obtained from the Bloomington *Drosophila* stock center.

Establishment of transgenic flies. To obtain transgenic fly lines with over-expression of dScgβ, the full-sequence of *dscgβ* was amplified from an expressed sequence tag (EST) clone, RE40051 (Berkeley *Drosophila* Genome Project), and cloned into the pUAST transformation vector using the *Not*I/*Xba*I site [16]. Plasmid pUAS-*dscgβ* contains a Flag tag sequence inserted at the N-terminus of dScgβ sequence.

To establish transgenic fly lines carrying *UAS-IR-dscgβ*, a 500bp fragment (5′-GACTTGGACTTTTGTCGCGACGAC to 5′-CGTCCGC AGTATAACATTCCGGGC) from the EST clone RE40051 was amplified, and then cloned into the *Not*I/*Avr*II site for the MCSIregion and the *Nhe*I/*Xba*I site for the MCSII region of the pWiz vector [17]. Thus we constructed a *dscgβ* inverted repeat (IR) plasmid head to head. The construct was verified by sequencing and then injected into embryos to obtain stable transformant lines carrying UAS-IR-*dscgβ*. P element-mediated germ line transformation was carried out as described earlier [18] and F1 transformants were selected on the basis of white eye color rescue [14]. Twelve transgenic strains carrying UAS-Flag-*dscgβ* constructs and five transgenic strains carrying UAS-IR-*dscgβ* constructs were established, respectively, and each transgenic strain showed consistent phenotype. To drive expression of *dscgβ* or *dscgβ* double-stranded RNA in animals, we crossed the transgenic flies with either the Act5C-Gal4 or the GMR-Gal4 line.

Generation of RNAi clones in retinae. RNAi clones in retinae were generated with the flip-out system [19]. Female flies with *hspFlp*; + ;*tub1 > FRT cd2 FRT > GAL4*,UAS-*GFP* were crossed with male flies with *w*; + ;UAS-IR-*dscgβ* and clones were marked by the presence of GFP expressed under the control of the tubulin promoter. Flip-out was induced 24–48 h after egg laying with a 60-min heat shock at 37 °C.

Production of rabbit anti-dScgβ polyclonal antibodies. Full-length *dscgβ* cDNA was amplified by PCR from a EST clone RE40051 using a forward primer including the *Eco*RI site (underlined), 5′-CCGGAATTC ATGATGCACACATTCGAGAACACC, and a reverse primer including the *Not*I site, 5′-ATAAGAATGCGGCCGCTTAGTTGATCTCGCAG GGATCGTC. The amplified fragment was digested with *Eco*RI and *Not*I, and subcloned into pGEX-6P-1 (Amersham Biosciences), an N-terminal glutathione-*S*-transferase (GST) fusion vector. A transformant of *Escherichia coli* BL21(DE3) was cultured to $A_{600} = 0.7$ at 37 °C and maintained in 1 mM isopropyl-β-D-thiogalactopyranoside at 25 °C for 4 h. The cells were sonicated and centrifuged, and then the supernatant was applied to a GSTrap column (Amersham Biosciences). Eluted GST-fused dScgβ protein was injected into a New Zealand White rabbit. After five booster injections, antiserum was applied to GST coupled Hitrap HP, then the flowthrough was applied to GST-fused dScgβ protein coupled Hitrap HP. Eluted antibodies against dScgβ were applied for Western immunoblot analysis and immunohistochemistry.

Western immunoblot analysis. Third instar larval extracts from Canton S and transgenic flies carrying Act5C-GAL4 > UAS-IR-*dscgβ* were applied to SDS–polyacrylamide gels containing 10% acrylamide and proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). The blotted membranes were blocked with TBS-T containing 2% bovine serum albumin, followed by incubation with polyclonal anti-dScgβ at 1:4000 dilution or monoclonal anti-α-tubulin (Developmental Studies Hybridoma Bank) at 1:2000 dilution for 16 h at 4 °C. After washing, the membranes were incubated with HRP-conjugated secondary antibodies (Amersham Biosciences) at 1:5000 dilution for 1 h at 25 °C. Detection was performed with ECL Western blotting detection reagent and images were analyzed with a Lumivision Pro HSIImage analyzer (Aisin Seiki).

Immunostaining. For immunohistochemistry, staged eyes were dissected, fixed in 4% formaldehyde, and blocked with 10% normal goat serum in PBST (PBS containing 0.15% Triton X-100). After blocking, the eyes were incubated in diluted primary antibodies in PBST with 10% normal goat serum for 16 h at 4 °C. The following antibodies were used; rabbit anti-dScgβ (diluted at 1:200), mouse anti-LacZ (1:500) (Promega),

mouse anti-dually phosphorylated ERK (1:200) (Cell Signaling Technology), rat anti-Elav (1:200), mouse anti-discs large (1:500), and mouse anti-βPS integrin (1:20) (Developmental Studies Hybridoma Bank). Eyes were washed three times with PBST and then incubated with secondary antibodies labeled with either Alexa546 or Alexa488 (Invitrogen) at 25 °C for 2 h. After three washes in PBST, eyes were mounted in mounting medium and analyzed by confocal laser scanning microscopy with a Zeiss LSM510.

Scanning electron microscopy. Adult flies were anesthetized, mounted on stages, and observed under a scanning electron microscope (SEM) VE-7800 (Keyence Inc.) in the low vacuum mode.

Histology of adult eyes. Adult eyes were fixed for 1 h in 2.5% glutaraldehyde/PBS, oxidized with 2% Osmiun tetroxide, and then dehydrated through a graded alcohol series (70%, 80%, 90%, 95%, and 100%, 15 min each). From 100% alcohol, the eyes were transferred to propylene oxide for 30 min, twice, then into a 1:1 mixture of propylene oxide/Durcupan resin for 2 h. Then, the eyes were placed in fresh resin and polymerized for 16 h prior to sectioning. Prepared sections were stained with 1% toluidine blue. The microscopic images were obtained by Olympus BX-50 microscope equipped with cooled CCD camera (Hamamatsu Photo.).

Results

Comparison of amino acid sequences between human and Drosophila β-sarcoglycans

Drosophila melanogaster has a smaller sarcoglycan family of proteins than mammals. The fly genome encodes a single orthologue of vertebrate α- and *ε-sarcoglycans* (*dscgα*), a *β-sarcoglycan* (*dscgβ*), and a single orthologue of *γ*-, *δ*-, and *ζ-sarcoglycans* (*dscgδ*) [12,20].

The amino acid sequences of dScgβ and human β-sarcoglycan (hScgβ) are shown in Fig. 1. dScgβ appears to share the same type II transmembrane characteristics as its human counterpart. The length of the leucine- and isoleucine-rich transmembrane region and their positions are also well conserved, along with four extracellular cysteine residues at the C-terminus, which are important in the production of functional proteins via intermolecular disulfide bonds [21]. The full-length sequences demonstrated only 19% identity and 35% similarity. The importance of the cysteine residues is demonstrated by the fact that a missense mutation in one conserved cysteine (C283Y) in γ-sarcoglycan is sufficient to cause LGMD type2C [22]. hScgβ has an EGF-like consensus and dScgβ has a similar sequence, though there is an extra residue between the second cysteine and the guanidine residue in the latter. dScgβ also has a sarcoglycan-1 domain which is conserved among internal regions of β-, δ-, and γ-sarcoglycans of the human, mouse, and rat (Fig. 1).

Drosophila Scgβ is required for eye development

In order to investigate the function of sarcoglycan complex in *Drosophila*, we examined the effect of the reduction of dScgβ protein in the retina using a combination of the GAL4-UAS targeted expression system and the RNAi method. We established five independent transgenic fly lines carrying UAS-*dscgβ*-IR. Knockdown of *dscgβ* in eye imaginal discs by the GMR-GAL4 driver strain, in which *dscgβ* double-stranded RNA is expressed in progenitor cells posterior to the morphogenetic furrow,

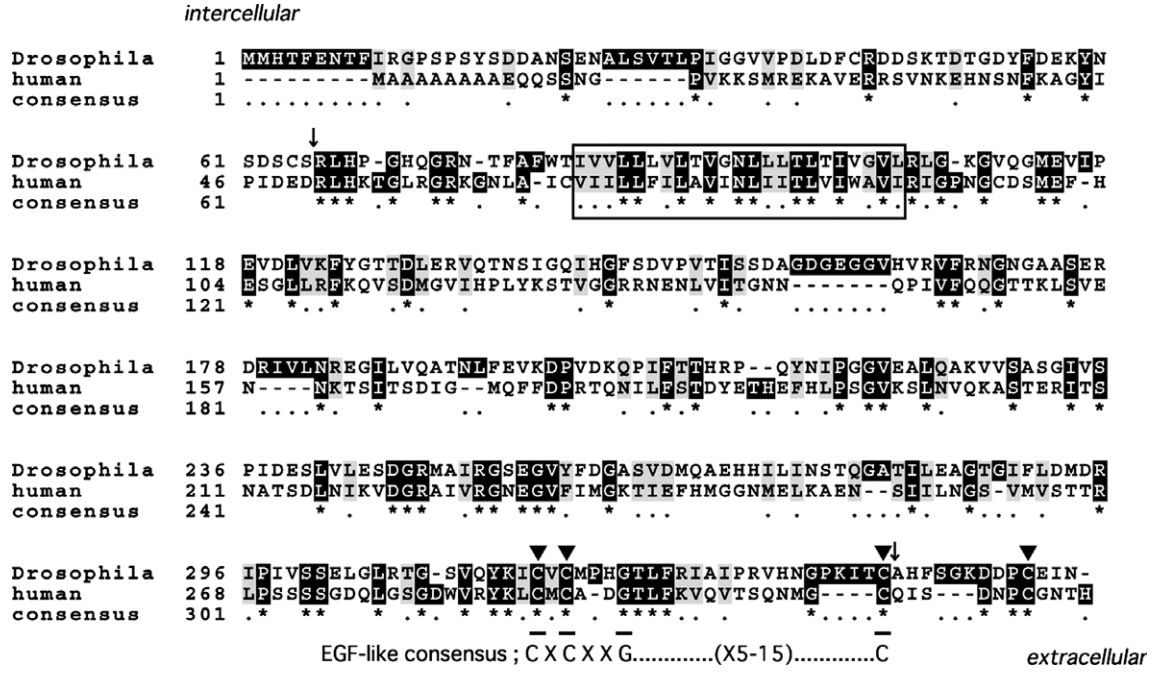

Fig. 1. Comparison of amino acid sequences between *Drosophila melanogaster* β-sarcoglycan (dScgβ) and human β-sarcoglycan (hScg*β*). Identical residues are highlighted in black and similar residues in gray. Putative transmembrane domains are indicated by the black box, and the start and terminal points of the conserved sarcoglycan-1 domain by the black arrows. The black arrowheads show the conserved four cysteine residues in the C-terminus, and the black underlining indicates the EGF-like consensus sequences. Alignment was performed with ClustalW at http://www.ebi.ac.uk/clustalw/ and highlighting was achieved with BOXSHADE at http://www.ch.embnet.org/software/BOX_form.html.

induced severe morphologically aberrant rough eyes. SEM images showed fusion of ommatidia and a lack of bristles (Fig. 2B, C, and F). Each independent transgenic fly strain showed essentially the same phenotype (Fig. 2B and C), suggesting that the rough eye phenotype is not induced by a possible insertional mutation. Regarding transgenic fly strains carrying UAS-IR-*dscgβ*, the strain number 72 was used throughout the following studies except for the experiment shown in Fig. 2C, in which the strain number 74 was used. Cross sections of the adult compound eyes, stained with toluidine blue, showed severe abnormality in the internal structure of the *dscgβ* knockdown flies (Fig. 2D and E). Especially, the height of the cells composing each ommatidium is much shorter than that of the control flies (Fig. 2D and E, lower panels). Flies carrying GMR-GAL4 alone exhibited apparently normal eye morphology (Fig. 2A) and over-expression of *dscgβ* exerted no significant effect (Fig. 2H).

To investigate whether the expression of *dscgβ* double-stranded RNA efficiently reduces the level of dScgβ protein, we conducted a Western immunoblot analysis with extracts of Act5C-GAL4 > UAS-*dscgβ*-IR larvae using an antibody against dScgβ. The level of dScgβ protein in the Act5C-GAL4 > UAS-*dscgβ*-IR fly line was reduced to 30.9% (Fig. 3A), as confirmed by immunostaining of retinae at 35 h after pupal formation (APF35) with anti-dScgβ antibodies. We utilized the flip-out system to produce the RNAi clone so that the level of dScgβ could be directly compared in single retinae. With the RNAi clone of *dscgβ*, marked by the presence of a GFP signal, dScgβ marked by Red was extensively reduced (Fig. 3B). To confirm the specificity of the RNAi effect,

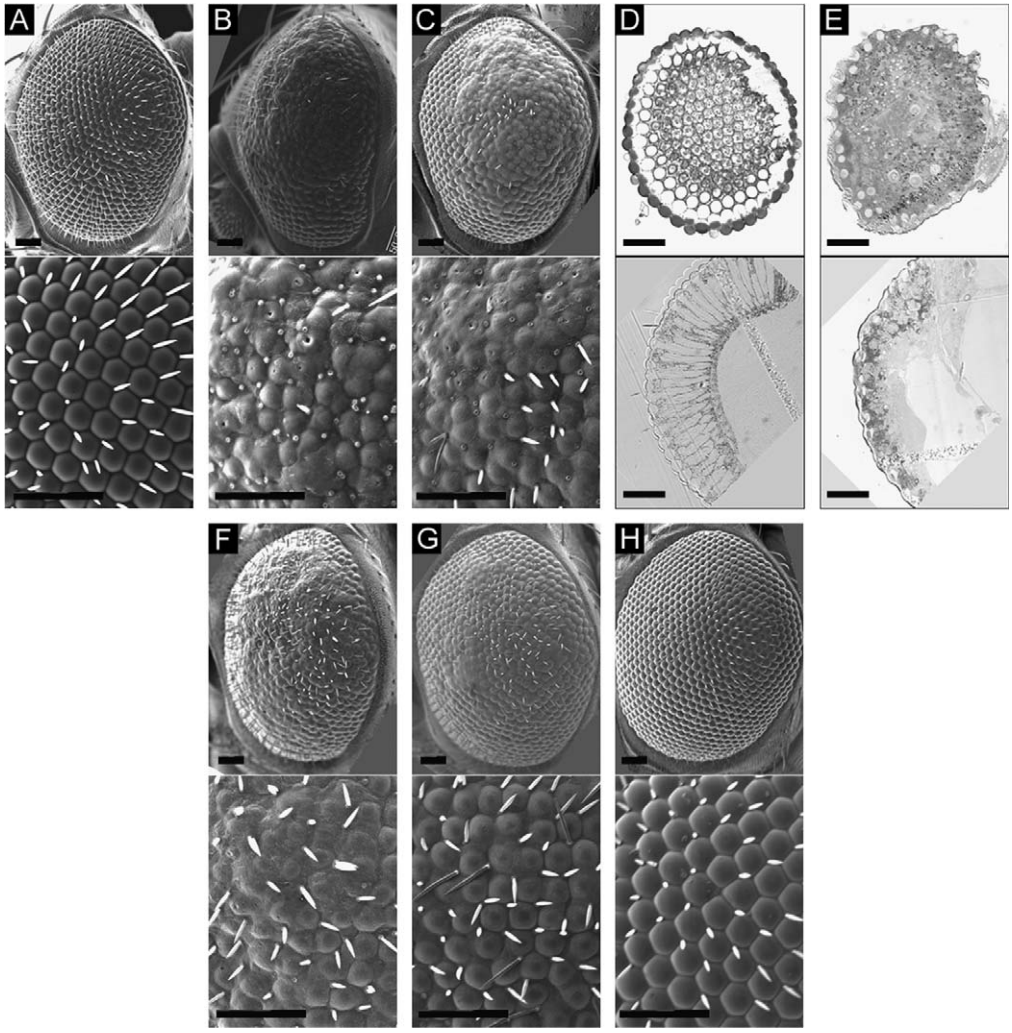

Fig. 2. Knockdown of *dscgβ* in the adult compound eyes induces the rough eye phenotype. (A–C) and (F–H) show scanning electron micrographs of adult compound eyes. The lower panels are higher magnifications of the images in the upper panels. (D,E) show crosssections of adult compound eyes stained with toluidine blue. The upper panels are horizontal sections and the lower panels are vertical sections. (A,D) GMR-GAL4/y;+;+. (B,E) GMR-GAL4/y;+;UAS-IR-*dscgβ*/UAS-IR-*dscgβ*. (C) GMR-GAL4/+;+;UAS-IR-*dscgβ*/+. (F) GMR-GAL4/+;+;UAS-IR-*dscgβ*/+. (G) GMR-GAL4/+;+;UAS-IR-*dscgβ*/UAS-*dscgβ*. (H) GMR-GAL4/+;+;UAS-*dscgβ*/+. Strain 72 carrying UAS-IR-*dscgβ* was used for (B and E–G) and strain 74 carrying the same transgene was used for (C). The flies were raised at 28 °C. Note that the rough eye phenotype is evident in (B–F). Over-expression of *dscgβ* rescued the rough eye phenotype induced by the knockdown of *dscgβ* (G). Strain 32 carrying UAS-*dscgβ* was used. The bars indicate 50 μm.

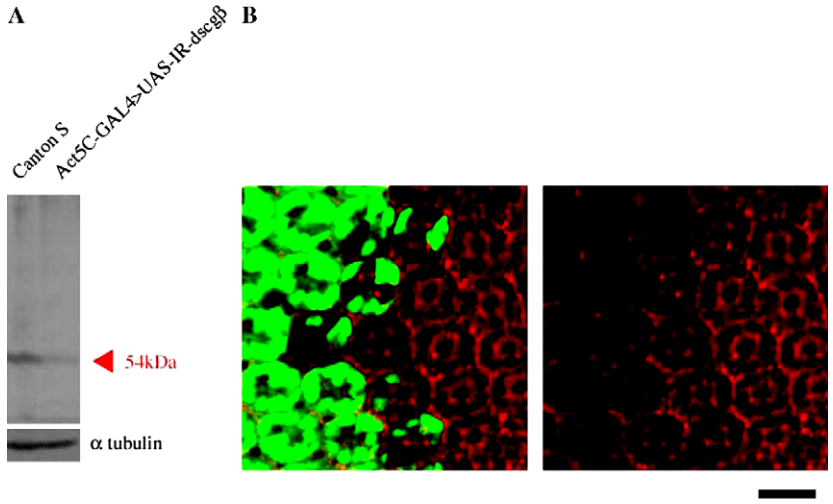

Fig. 3. Specificity of anti-dScgβ antibody. (A) Western immunoblot of third instar larvae protein extracts from Canton S (left lane) and UAS-IR-*dscgβ*/Act5C-GAL4 (right lane) flies. The blots were probed with anti-dScgβ and anti-α tubulin antibodies. (B) The *dscgβ* double-stranded RNA is positively marked with GFP, shown in green. In the RNAi clone area, the level of anti-dScgβ signals is reduced. The bar indicates 33.3 μm.

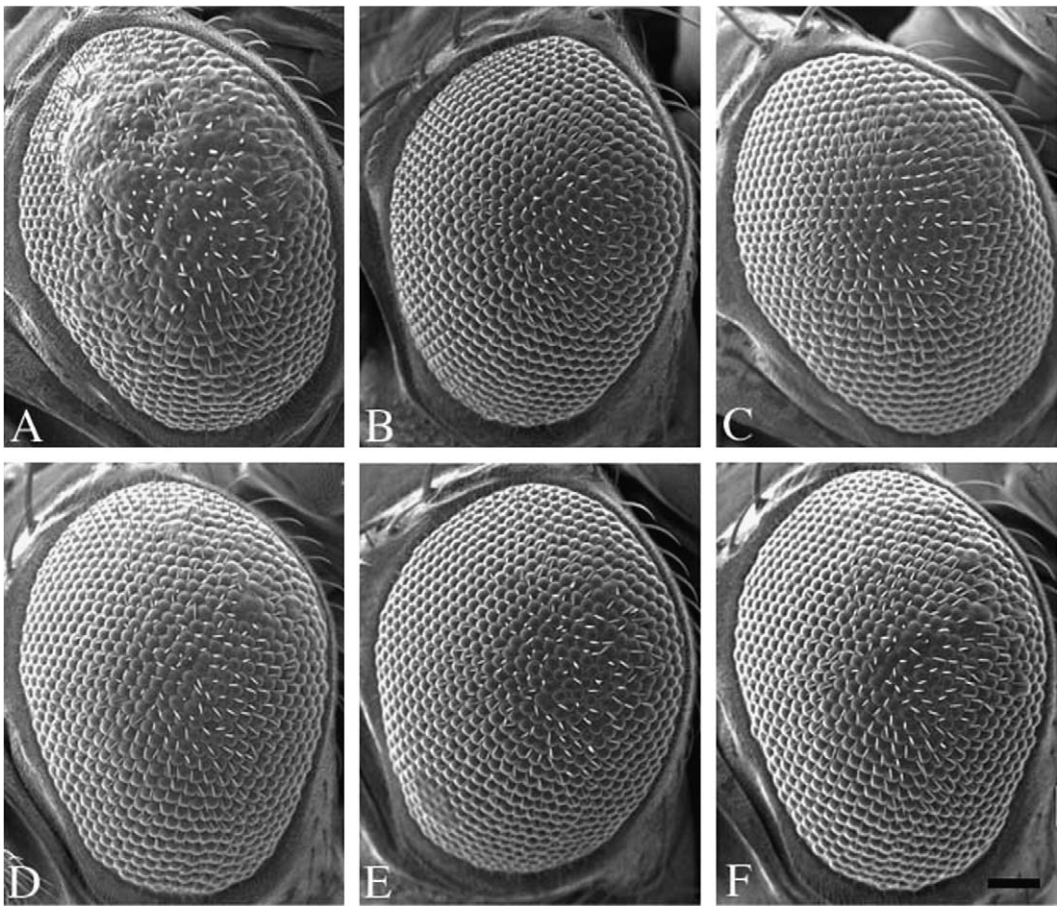

Fig. 4. Scanning electron micrographs of adult compound eyes. Female flies expressing *dscgβ* double-stranded RNA (GMR-GAL4/GMR-GAL4;+;UAS-IR-*dscgβ*/UAS-IR-*dscgβ*) were crossed with $mirror^{SaiD3}$ (loss of function), ru^{1} (hypomorph), rho^{ve-1} (loss of function), rho^{7M43} (amorph) or rho^{AA69} (undetermined) male flies, and then F1 progeny was developed at 28 °C without balancer chromosomes and used for inspection of eye phenotype. (A) GMR-GAL4/+;+;UAS-IR-*dscgβ*/+. (B) GMR-GAL4/+;+;UAS-IR-*dscgβ*/$mirror^{SaiD3}$. (C) GMR-GAL4/+;+;UAS-IR-*dscgβ*/ru^{1}. (D) GMR-GAL4/+;+;UAS-IR-*dscgβ*/rho^{ve-1}. (E) GMR-GAL4/+;+;UAS-IR-*dscgβ*/rho^{7M43}. (F) GMR-GAL4/+;+;UAS-IR-*dscgβ*/rho^{AA69}. The bar indicates 50 μm.

GMR-GAL4 > UAS-*dscgβ*-IR flies were crossed with flies carrying UAS-*dscgβ*. Over-expression of *dscgβ* at least partially rescued the rough eye phenotype induced by knockdown of *dscgβ* (Fig. 2G). The full-length amino acid sequence of dScgβ has low identity with that of dScgα (12.2%) and dScgδ (13.6%). Furthermore, the cDNAs of *dscgα*, *dscgβ*, and *dscgδ* share less than seven sequential nucleotides with each other. Therefore, it is unlikely that the expression of *dscgβ* double-stranded RNA affects the expression of *dscgα* and *dscgδ* genes. These results indicate that the rough eye phenotype observed in the RNAi flies of *dscgβ* is due to reduction of the dScgβ protein level.

Genetic link between dscgβ and rhomboid

Recently, the DGC has been suggested to contribute to cellular communication, as indicated by the possible interactions with known signaling molecules such as nitric oxide synthase [23], voltage-gated sodium channels [24,25] or Grb2 [26,27]. It has been reported that dystroglycan can act antagonistically to other adhesion molecules, such as integrins, resulting in suppression of the downstream ERK-MAPK cascade [28,29]. A more recent report described γ-sarcoglycan deficiency to increase the MAPK pathway in mouse cultured myotubes [9]. We therefore examined the effects of mutations that modify the dScgβ-induced rough eye phenotype, especially focusing on genes related to signal transduction.

Half dose reduction of the Egfr signaling related gene *rhomboid-1* significantly suppressed the rough eye phenotype (Fig. 4). On the other hand, no modification of the rough eye phenotype was observed with half reduction of the JNK signaling related genes *bascket*, *hemipterous*, Wnt signaling related gene *wingless*, or Hedgehog signaling

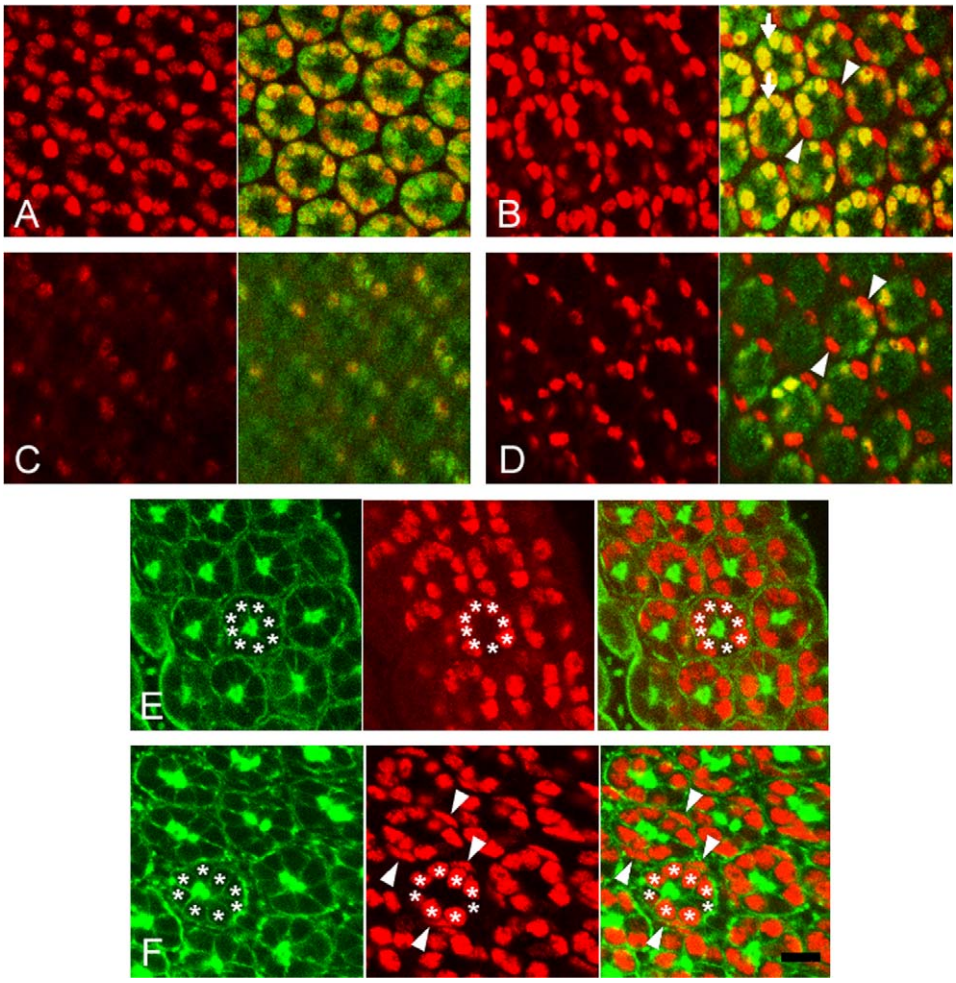

Fig. 5. Extra cells are recruited in 35APF retinae of *dscgβ* knockdown flies. (A, C, and E) GMR-GAL4/+;+;+/X63. (B, D, and F) GMR-GAL4/+;+;UAS-IR-*dscgβ*/X63. (A–D) Red and green indicate anti-lacZ and anti-Elav signals, respectively. Anti-lacZ signals are shown in left panels and the merged images of anti-lacZ and anti-Elav signals in right of (A–D). Confocal sections of the middle phase of the retinae (A,B), and the upper phase of the retinae (C,D) are shown. (E,F) Red (middle) and green (left) indicate anti-lacZ and phalloidin bound Actin signals, respectively. The merged images are shown in the right of (E,F). The white arrowheads in (B-right and D-right) indicate examples of extra cells expressing Rhomboid, the white arrows in (B-right) indicate the examples of ommatidia with photoreceptors ectopically expressing Rhomboid strongly. The extra cells do not co-express Elav. Asterisks in the (E,F) mark eight photoreceptor cells in a ommatidium. The distinct rhomboid positive extra cells indicated by the white arrowheads are evident (F-middle and right). The bar indicates 10 μm.

related gene *hedgehog* (data not shown). All three different alleles of *rhomboid-1* showed suppression of the rough eye (Fig. 4D–F). Rhomboid-1 is a seven membrane-spanning serine protease, undergoing cleavage of Spitz to release the secreted form as Egfr ligand from the Golgi apparatus [30]. In the eye, Rhomboid-3, also known as Roughoid, cooperates with Rhomboid-1 as rate-limiting components of Egfr signaling [31]. Expectedly, the Rhomboid-3 hypomorph mutant ru^{1} demonstrated suppression of the rough eye phenotype induced by knockdown of *dscgβ* (Fig. 4C). Moreover Mirror loss of function mutant $mirr^{SaiD3}$ also suppressed the rough eye phenotype (Fig. 4B). The *mirror* gene encodes a homeodomain-containing transcription factor that is thought to activate transcription of *rhomboid* [32]. These results suggest a genetic link between dScgβ and the Egfr signaling molecules.

Knockdown of dscgβ induces ectopic expression of Rhomboid, which causes activation of ERK

The observation that mutations in *rhomboid* suppress the rough eye phenotype induced by knockdown of *dscgβ* suggests that expression or activation of Rhomboid might be perturbed in the eyes of dScgβ RNAi flies. Therefore, we examined this question using an enhancer trap line, X63, in which *lacZ* is inserted near the *rhomboid* gene in the third chromosome so that *rhomboid* promoter activity can be monitored by *lacZ* expression. In third instar larval eye imaginal discs, no differences in *rhomboid-lacZ* expression were observed between control flies and dScgβ RNAi flies (data not shown). This might be due to the relatively slow turn over rate of dScgβ protein. Maternally stored dScgβ protein might persist until the larva stage (data not shown). On the other hand, ectopic *rhomboid-lacZ* expression was observed in pupal retinae at 35APF (Fig. 5A–F). At this stage, reduction of dScgβ protein in retina is evident in dScgβ RNAi flies (Fig. 3B). Each ommatidium has two additional cells expressing *rhomboid-lacZ* around the eight photoreceptor cells (Fig. 5A and B). These two cells are more clearly seen in the images of the upper phase (Fig. 5C and D). Double staining with anti-lacZ and phalloidin further confirmed that these cells are ectopic extra cells since eight original photoreceptor cells and additional two cells expressing *rhomboid-lacZ* strongly are clearly seen in each ommatidium (Fig. 5E and F). In contrast, neither *seven-up-lacZ* nor *rough-lacZ* was expressed in these extra cells, indicating the ectopic expression to be specific for Rhomboid (Fig. 6). These cells did not appear to be destined to become photoreceptor cells, since they were found to lack Elav, a marker of differentiated neurons (Fig. 5B). In addition, *dscgβ* knockdown flies showed strong *rhomboid-lacZ* expression not only in the R2/R5/R8 typical of wild-type flies but also in other photoreceptor cells (Fig. 5A and B).

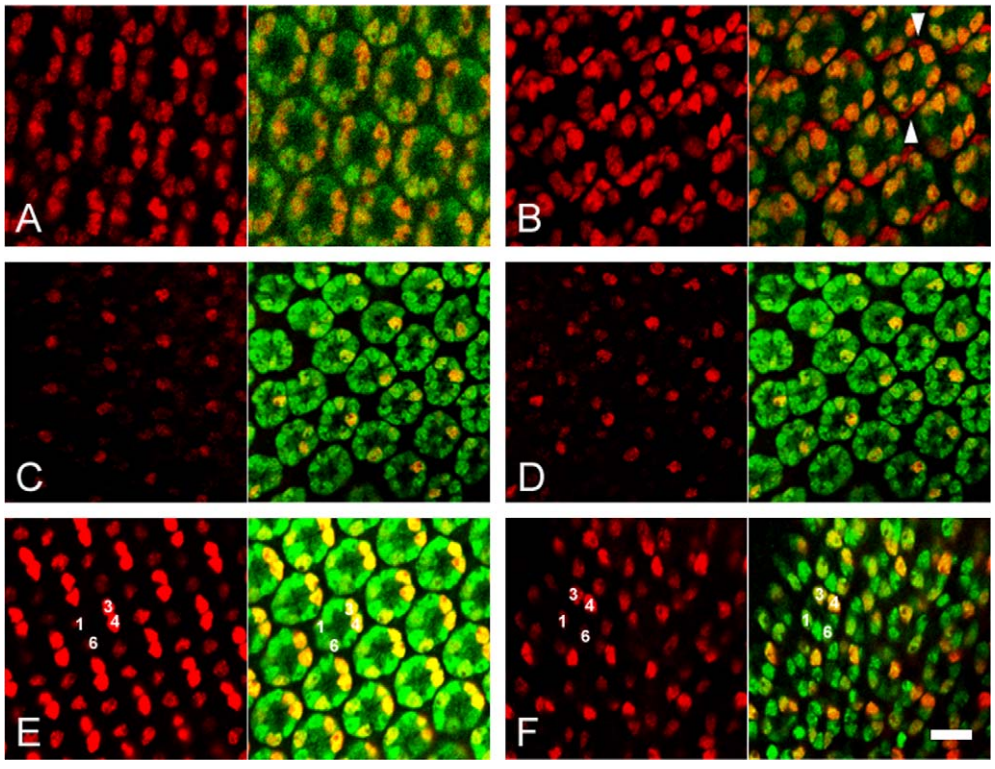

Fig. 6. Ectopic expression in the extra cells is specific for Rhomboid. Confocal sections, stained with anti-lacZ (red) and anti-Elav (green). Anti-lacZ signals are shown in left panels and the merged images of anti-lacZ and anti-Elav signals in right panels. (A) GMR-GAL4/+;+;+/X63. (B) GMR-GAL4/+;+;UAS-IR-*dscgβ*/X63. (C) GMR-GAL4/+;+;+/ro156. (D) GMR-GAL4/+;+;UAS-IR-*dscgβ*/ro156. (E) GMR-GAL4/+;+;+/AE127. (F) GMR-GAL4/+;+;UAS-IR-*dscgβ*/AE127. X63, ro156, and AE127 have lacZ insertions in *rhomboid*, *rough*, and *seven-up*, respectively. The white arrowheads in panel B-right indicate examples of extra cells expressing Rhomboid. The numbers in the (E,F) indicate the photoreceptor cells. The bar indicates 10 μm.

As described above, Rhomboid enhances Egfr signaling and activation of ERKA [33–35]. Therefore, we next examined whether ERK is phosphorylated. Immunostaining data using antibodies against dpERK demonstrated activation in both the extra cells and the photoreceptor cells ectopically expressing rhomboid (Fig. 7). This activation pattern of ERK perfectly corresponded with the rhomboid expression pattern in retinae of *dscgβ* knockdown flies. These results, taken together, indicate that the rough eye phenotype in *dscgβ* knockdown flies is derived from ectopic rhomboid expression causing activation of ERKA via Egfr signaling.

Proper localization of βPS Integrin in the retina of late pupal developmental stages is disrupted in the dscgβ knockdown flies

To clarify the process that directs such a severe rough eye phenotype in the adult with the knockdown of *dscgβ* (Fig. 2B–D), we investigated the structure of the pupal retina at late stage. Immunostaining with anti-discs large antibody revealed that each cell in 50APF pupal retina of *dscgβ* knockdown flies is attached loosely, the orientation of ommatidia is irregular, and the size of ommatidia varies (Fig. 8A and B). We then examined the localization of βPS Integrin, since it is well known to function in cell-matrix and cell–cell adhesion [36]. Furthermore, in mammal, it is reported that mammalian Sarcoglycan interacts with Integrins [5,7,8]. In the 40APF retinae of the wild-type flies, βPS Integrin is strongly expressed in the focal adhesion sites of the cone cell feet to the retinal ECM and weakly in those of the pigment cell feet to the retinal ECM

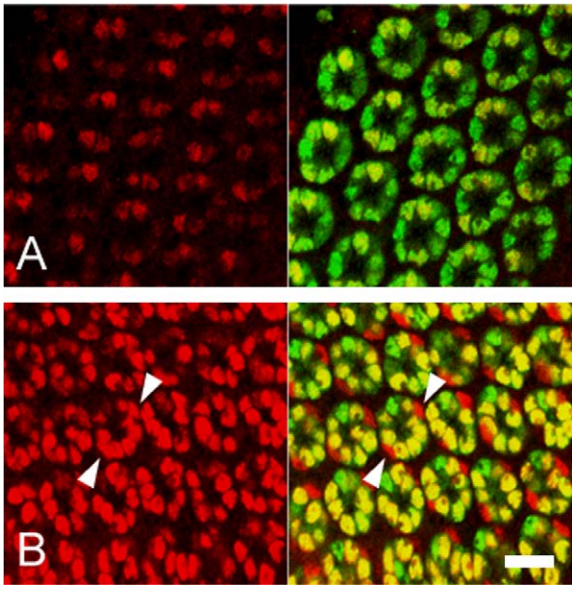

Fig. 7. Activation of ERK. Anti-dpERK signals (red) are shown in left panels and the merged images of anti-dpERK (red) and anti-Elav (green) signals in right panels. In the 35APF retinae of *dscgβ* knockdown flies (B), ERKA is phosphorylated both in the extra cells (white arrowheads) and in the photoreceptor cells that do not exhibit phosphorylation of ERK in the control flies (A). The bar indicates 10 μm.

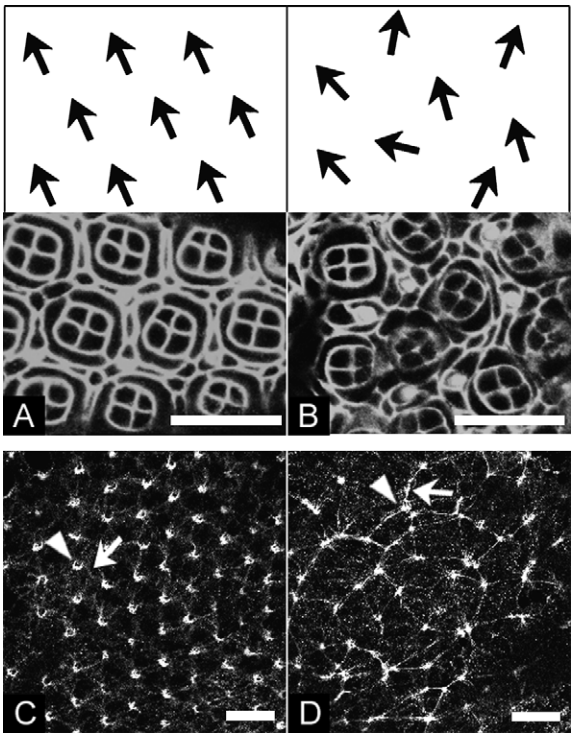

Fig. 8. The effect of *dscgβ* knockdown on the morphogenesis of late pupal developmental stages' retinae. Confocal sections stained with anti-discs large (lower panels of A and B) and anti-βPS Integrin (C,D). (A,C) control flies. (B,D) *dscgβ* knockdown flies. Black arrows in the upper panels of (A,B) indicate the orientation of the ommatidia. Note that the orientation of the ommatidia is disrupted in the *dscgβ* knockdown flies (upper panel B). The size of ommatidia is also irregular, and the cell attachment is loose (compare lower panels of A and B). The cone cell feet bundles and the pigment cell feet are shown by white arrowheads and arrows, respectively (C,D). Disruption of βPS Integrin distribution pattern is seen in (C,D). The bars indicate 20 μm.

(Fig. 8C). On the other hand, in the 40APF retinae of the knockdown flies of *dscgβ*, much stronger βPS Integrin signal in the pigment cell feet was observed (Fig. 8D). It is also apparent that the distribution of the cone cell feet bundles is irregular in the retinal floor (Fig. 8D). These observations suggest that multiple effects induced by knockdown of *dscgβ* cause eventual rough eye phenotype in the adult compound eyes.

Discussion

Previous studies have suggested that the DGC has signaling functions in addition to playing a structural role. First, Syntrophin, one of the components of DGC, binds to PIP2 [37], neuronal NO synthase [23], calmodulin [38,39], and Grb2 [26,27]. Second, Dystroglycan interacts with several components of the ERK-MAP kinase cascade, and modulates ERK activity in response to the binding of α6β1 integrin to laminin [29]. Third, γ-sarcoglycan deficiency in mouse appears to activate ERK [9]. Despite many studies about the possible involvement of DGC in the signaling pathways, however, the definitive mechanisms in regulation of signaling pathways by sarcoglycan remain

to be elucidated. Clarification should provide clues to understanding the etiology of Limb girdle muscular dystrophy.

We here report first findings of interactions between Sarcoglycan and Rhomboid, a rate-limiting component of the Egfr signaling pathway. In the eyes of the flies with knockdown of *dscgβ*, each ommatidia has two extra cells strongly expressing rhomboid, causing activation of ERKA. Hitherto, it has been reported that over-expression of Rhomboid in the eye induces one, two or occasionally three extra cells, identified as the mystery cells which eventually transform into photoreceptors expressing neural antigens, possibly R3 and R4 [40]. Therefore, the two extra cells in the *dscgβ* knockdown ommatidia are likely mystery cells failing to be eliminated.

Loss of function alleles of Egfr or its ligand Spitz do not suppress the rough eye phonotype induced by knockdown of *dscgβ* (data not shown). However, these contradictory results might be explained as follows. Once activated, the signaling cascade could be amplified progressively, so that only a half reduction of downstream components of the signaling may not be sufficient to suppress the effects of over-activation of the initiator. In any event, the present study indicates that the sarcoglycan complex including dScgβ negatively regulates Egfr signaling. What is the mechanism for this negative regulation? dScgβ has an EGF-like sequence in its C-terminal extracellular domain (Fig. 1) which might mask the ligand-binding domain of Egfr and consequently sarcoglycan might function antagonistically.

Egfr signaling is required for triggering determination of non-R8 photoreceptors, this being initiated by Rhomboid, which allows the release of Spitz, the Egfr-activating ligand. Appropriate levels of Egfr signaling are required for correct ommatidial rotation, since defects in the latter are seen under conditions in which Egfr signaling is either increased or decreased. Furthermore, it has been reported that the eyes of Egfr temperature-sensitive mutant allele $Egfr^{ts1}$ lack photoreceptors in some clusters, and show occasional misrotation of their ommatidia [41]. Conversely, in $argos^{rlt}$ mutants in which Egfr signaling is increased because of half reduction of the antagonistic effect of argos against Egfr signaling, most mystery cells are transformed into R3/R4 photoreceptor cells though they fail to develop into neurons ultimately, which could cause spatial disruption and perturb the normal ommatidial rotation [41]. Correspondingly, our examination of pupal eyes revealed that knockdown of *dscgβ* results in extra cells like mystery cells with the ability to remain alive and strongly expressing rhomboid, even after the time when they should have disappeared (Fig. 5). It also revealed disruption of the correct orientation of most ommatidia (Fig. 8A and B). These findings suggest that similar molecular events occur with both up-regulation of Egfr signaling and knockdown of *dscgβ*. In the present study, the fact that *dscgβ* induced phosphorylation of ERK via activation of Egfr signaling (Fig. 7) is consistent with the previous observation that phosphorylation of ERK2 is increased constitutively in the extensor digitorum longus muscle of γ-sarcoglycan null mice[9].

Several possible mechanism can be considered how extra cells relate to the severe rough eye phenotype observed in the *dscgβ* knockdown flies. One possible explanation is that the existence of the extra cells disrupts the proper spatial distribution of the surrounding cells, causing the disruption of βPS Integrin distribution pattern at the retinal floor in the later pupal developmental stages, and finally resulted in the rough eye phenotype in the *dscgβ* knockdown adult flies. An alternative explanation is that the existence of the extra cells only partially contributes to the rough eye phenotype, independent of the disruption of βPS Integrin distribution pattern. As described above, Integrins and Sarcoglycans are known to have an interaction in mammal [5,7,8]. It is therefore possible that the disruption of βPS Integrin distribution pattern in the *dscgβ* knockdown flies is independent of the appearance of extra cells in the early developmental stages of pupae. In addition, expression of p35 [42] or DIAP2 [43], inhibitors of apoptosis, at least partially suppressed the rough eye phenotype induced by the knockdown of *dscgβ* (data not shown), suggesting that the apoptosis induced by *dscgβ* during eye development at least partially contributes to the rough eye formation. Taken together, several possible mechanisms and their combination likely contribute to the severe rough eye phenotype observed in the adult fly.

In summary, we have shown that knockdown of *dscgβ* in the *Drosophila* retina induces a rough eye phenotype with production of extra cells, a defect of ommatidial orientation, and the disruption of βPS Integrin distribution pattern at the retinal floor. In addition, over-expression of *rhomboid* results in activation of Egfr signaling and phosphorylation of ERK. Here, we provide the first definitive evidence that the sarcoglycan complex plays an important role in regulation of the Egfr signaling cascade. Moreover, the *dscgβ* knockdown flies established in this study should provide a useful tool for determining novel interactants with *dscgβ*.

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