Sarcoglycan complex formation is involved in regulation of EGFR signaling during Drosophila eye development

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Abstract

**Background:** Mutations of some components of the Dystrophin Glycoprotein Complex (DGC) are known to cause Muscular Dystrophy in humans. An innovative approach to the further understanding of pathological mechanisms and physiological roles of the DGC has been recently made using several non-mammalian animal models, using species such as Caenorhabditis elegans, Xenopus laevis, and zebrafish. The mammalian DGC is in most part conserved in Drosophila, with respect to the amino acid sequence. However, detailed comparisons of the characteristic properties of each DGC components between mammals and Drosophila have yet to be made.

**Methods:** In the present study, we analyzed mutual binding ability of Drosophila Sarcoglycans in order to address the possibility of complex formation, a characteristic of the mammalian counterparts.

**Results:** In vitro binding assays showed that Drosophila Sarcoglycans could mutually bind to each other. Moreover, β-Sarcoglycan and δ-Sarcoglycan could directly associate with Epidermal growth factor receptor (EGFR) via the EGF-like consensus sequences of their C-terminus regions.

**Conclusions:** In vitro data in combination with immunohistochemical data for sarcoglycan knockdown fly retinae, we propose that Sarcoglycan complex formation is required for eye development. Notably, the direct binding of β-Sarcoglycan and δ-Sarcoglycan to EGFR might be critical for the proper regulation of EGFR signaling during eye development in Drosophila.

**Keywords:** Sarcoglycan, Drosophila, EGFR, muscular dystrophy, eye development.

Background

The Dystrophin Glycoprotein Complex (DGC) stabilizes the plasma membrane structure, especially during cell movement, by linking the internal cytoskeleton and the extracellular matrix. It is basically a heterogeneous large complex consisting of various combinations of Dystrophin, Utrophin, Dystroglycan, Sarcoglycans, Syntrophins, Dystrobrevins and Sarcospan in mammals [1]. The six forms of Sarcoglycan (α-, β-, γ-, δ-, ε- and ζ-Sarcoglycan) in mammals are each encoded by independent gene loci [2-7]. Mutations in α-, β-, γ- and δ-sarcoglycan are reported to cause limb girdle muscular dystrophies (LGMD) [8,9]. 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 Drosophila α-Sarcoglycan (dScgα), β-Sarcoglycan (dScgβ), and δ-Sarcoglycan (dScgδ) correspond to α-ε-Sarcoglycan, β-Sarcoglycan and γ-δ-ζ-Sarcoglycan in human, respectively [10,11]. δ-Sarcoglycan deficient mutant flies have been described to show progressive muscle dysfunction, mobility defects and reduced life span [12].

One of the proposed functions of the Sarcoglycan complex is to stabilize DGC itself by interacting with other constitutive proteins [13-16]. The β-/δ-Sarcoglycan core is known to interact with the C-terminus of dystrophin [13]. Biochemical preparations from sarcoglycan mutant muscle have revealed less tightly adherent α-dystroglycan subunits suggesting abnormal interaction in the absence of Sarcoglycan [15-17]. However, from other studies [18], one can suggest that Sarcoglycans might also have non-mechanical functions, for example functionally compensating for integrins in muscle. It has also been reported that sarcoglycans associate with integrins and nNOS, indicating a potential role in signaling transduction. In fact, we reported possible interactions between dScgβ and Epidermal Growth Factor Receptor (EGFR) signaling in Drosophila, from genetic screening and immunohistochemical analyses of dscgβ knockdown pupal retina [19].

Despite relatively conserved homology based on amino acid sequence comparisons [10], concrete evidence confirmed by experimental results of characteristic and functional similarities of DGC components between mammals and Drosophila is still missing. Therefore, in this study, we performed in vitro binding assays of three Drosophila Sarcoglycans, dScgα, dScgβ and dScgδ. In practice, complex formation of Sarcoglycan subunits is necessary to exert its contribution to the biological roles of the DGC in mammals [13,20].
It is commonly assumed that defects in a single Sarcoglycan bring about severe impairment of the appropriate assembly. In turn, the generation of aberrant protein aggregation interferes with the normal function [21]. In our previous report, over-activation of EGFR signaling was detected in dscgβ knockdown fly retina, presumably due to ectopic expression of rhomboid, encoding the protease that activates positive EGFR ligands [19]. In the present study, we demonstrate that knockdown of other Sarcoglycan family genes, dscga and dscgδ also induced ectopic expression of rhomboid in the pupal retinae as observed in the knockdown flies of dscgβ. Moreover, in vitro binding assays of recombinant Sarcoglycans and EGFR revealed direct associations via the highly conserved EGF-like consensus sequences present in the C-termini of dScgβ and dScgδ. Collectively from our findings, we propose that: (1) characteristic functions of Sarcoglycans in terms of the complex formation are conserved between Drosophila and mammals; (2) Sarcoglycan complex formation is required for negative control of EGFR signaling; and (3) direct association of dScgβ and dScgδ with EGFR plays a significant role in the regulation of EGFR signaling during Drosophila eye development.

Methods
Cloning of target genes into the pETDuet-1 vector
For co-expression of two target genes in Escherichia coli (E. coli), we used the pETDuet-1 vector which contains two multiple cloning sites (Novagen). 6×His was tagged for one of the expressed proteins while Flag tag was designed for another. All the genes inserted into the pETDuet-1 vector were amplified by PCR using KOD plus (Toyobo). The primer pairs and the DNA resources for the PCR reactions, and the restriction enzymes used for the cloning into the pETDuet-1 vector are summarized in Table 1. cDNAs containing EST clones, RH11377 and RE40051, were obtained from Berkeley Drosophila Genome Project, and cDNAs derived from an Oregon R adult female were obtained from mRNA with polyA tails by RT-PCR. For the cloning into multiple cloning site 1 (His-tag) of the pETDuet-1 vector, the PCR products obtained from the cDNA resources were directly inserted at the distinct restriction enzyme sites of a blank pETDuet-1 vector. For cloning of Flag-tagged target genes into the multiple cloning site 2 of the pETDuet-1 vector, the initial PCR products from the cDNA resources were first sub-cloned downstream of the flag tag sequence in a modified pUAST vector in order to add a Flag tag. Then, the target genes with flag sequences were amplified by secondary PCR reactions using the sub-cloned pUAST vectors as DNA templates and inserted into the multiple cloning site 2 of the plain pETDuet-1 vectors or vectors carrying His tagged counterpart target genes to obtain the final objective constructs. Notably, no tag was added to Lamin, since a well-working antibody against Lamin was commonly available from the Developmental Studies Hybridoma Bank (DShB). A 1677 base pair (bp) fragment encoding 1-559 amino acids of Drosophila EGFR PA was chosen for the truncated form of Drosophila EGFR (Egfr1-559), while a 942bp fragment encoding 1-314 amino acids of dScgβ and a 1131bp fragment corresponding to 1-377 amino acids of dScgδ were employed for the EGF consensus sequence deletion forms of dScgβ (dScgβΔEGF-like) and dScgδ (dScgδΔEGF-like), respectively. The obtained protein products concerning Sarcoglycans in this experiment are schematically represented with rectangular bars in Figure 1. All the constructs were verified by nucleotide sequencing before use.

Table 1-Cloning into pETDuet vector.
All the used primer pairs, restriction enzymes and DNA resources of the generated constructs for in vitro binding assay using pETDuet vector are listed. F’ and R’ respectively represent forward and reverse primers. The sequences of the used restriction enzyme sites are underlined.
In vitro protein binding assay

Expression of the target genes was induced by 1mM isopropyl-β-D-thiogalactopyranoside (Wako) with incubation for 2 h at 37°C in *E. coli* BL21DE3. The *E. coli* were then sonicated in Wash buffer (20mM Tris-HCl (pH 8.0) containing 10% glycerol, 0.5M NaCl, 50mM Imidazole, 20mM β-mercaptoethanol, and 0.5% Tween-20, 0.5% TritonX-100). The Input samples and the elution fractions were applied to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblot analysis. The proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) after SDS-PAGE, and the blotted membranes were blocked with TBS-T containing 2% blocking reagents for 1h at 25°C followed by incubation with mouse anti-His (Invitrogen) at 1:2,000 dilution, mouse anti-Flag (Sigma) at 1:4,000 dilution, or mouse anti-Lamin (DSHB) at 1:8,000 dilution for 16h at 4°C. After washing, the membranes were incubated with HRP-conjugated secondary antibodies (GE healthcare) at 1:100,000 dilution for 1h at 25°C. Detection was performed with ECL Western blotting detection reagent (GE healthcare) and images were analyzed with a Lumivision Pro HSII image analyzer (Aisin seiki).

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. An enhancer trap line carrying the lacZ marker rho<sub>663</sub> (<em>rhomboid</em>) was obtained from Y. Hiromi, while others carrying lacZ markers sp3<sup>1084</sup> (<em>spitz</em>) and argos<sup>05845</sup> (<em>argos</em>) and rho<sup>1</sup> were obtained from the Bloomington *Drosophila* stock center. Establishment of lines carrying GMR-GAL4 was described earlier [22].

Establishment of transgenic flies

In order to establish transgenic fly lines applicable for knockdown, two independent regions for each *sarcoglycan* were designed to construct plasmids with hairpin-loop structures. The plasmid constructs were named α<sub>i</sub> for *dscgα* knockdown, β<sub>i</sub>, β<sub>i</sub> for *dscgβ* knockdown and δ<sub>i</sub>, δ<sub>i</sub> for *dscgδ* knockdown, respectively. Notably, since we have already established transgenic flies carrying UAS-Inverted Repeated (IR)-*dscgβ* whose target region corresponds to β<sub>i</sub> in our previous work [19], we used these original lines for UAS-IR-β<sub>i</sub> in this study. Therefore, concerning *dscgβ* knockdown, we only generated transgenic flies carrying UAS-IR-dscgβ<sub>i</sub> whose target region is not overlapped with UAS-IR-dscgβ<sub>i</sub> in this study. The employed target regions are schematically shown in Figure 1 for easy comparison and the cloning information is summarized in detail in Table 2. All the genes inserted into the pWIZ vector were obtained from EST clones or cDNAs derived from Oregon R adult females by PCR using KOD plus (Toyobo). NHL/ Xbal sites were inserted for insertion of target genes into multiple cloning site 1, while Nhel/ EcoRI fragments were used for compatible cloning into Avrl/ EcoRI sites of multiple cloning site 2. Thus we constructed α<sub>i</sub>-IR, α<sub>i</sub>-IR, β<sub>i</sub>-IR, δ<sub>i</sub>-IR, and δ<sub>i</sub>-IR plasmids head to head. The

Figure 1-The employed constructs and alignment of the conserved EGF-like consensus sequences.

(A) Diagrams of the target region of the constructs for the generation of RNAi flies and for the recombinant proteins for in vitro binding assays. Rectangular bars show the obtained recombinant proteins. Gray areas of the bars represent the EGF-like consensus region. (B) Details of amino acid sequences of the conserved EGF-like sequences are highlighted in gray and the essential cysteine and glycine residues in black in panel B. X can be any arbitrary amino acids. In the C-terminus region, dScgβ and dScgδ possess an EGF-like consensus sequence, which is characteristic of EGFR ligands such as human TGFα and Spitz (*Drosophila* homologue of TGFα). This sequence is also conserved in mammalian Sarcoglycans except α- and ε-Sarcoglycan. The numbers written below the black-highlighted cysteine (C) indicate the amino acid numbers counted from the N-terminus.
constructs were verified by sequencing and then injected into embryos to obtain stable transformant lines carrying UAS-IR-dscgα i1, UAS-IR-dscgα i2, UAS-IR-dscgβ i2, UAS-IR-dscgδ i1, and UAS-IR-dscgδ i2. P element-mediated germ line transformation was carried out as described earlier [23] and F1 transformants were selected on the basis of white eye color rescue [24]. The established transgenic strains are summarized in Table 3. Five, four, eleven, eight and thirteen transgenic strains carrying UAS-IR-dscgα i1, UAS-IR-dscgα i2, UAS-IR-dscgβ i2, UAS-IR-dscgδ i1, and UAS-IR-dscgδ i2 were established, respectively. To drive expression of the double-stranded RNAs of dscgα, dscgβ or dscgδ in animals, we crossed the transgenic flies with the GMR-GAL4 line. Not all the strains showed a similar extent of the rough eye phenotype, probably due to differences in RNAi efficiency, but at least three strains for each construct displayed a consistent rough eye phenotype.

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

mRNA preparation and semi-quantitative RT-PCR
Total RNA was isolated from the third instar larvae by TRIzol (Invitrogen) followed by DNaseI treatment. mRNA was purified using an Oligotex™-dT30 <super>mRNA purification kit (Takara). Then, cDNA was obtained using oligo (dT) primer and PrimeScript™
RTase (Takara). The following primers were used for the gene amplification: For dScgα, 5'-AGTTCAGGGTCTAAAGTTGCA-3' and 5'-AAAGCTGCCCACGTGGACAAT-3', for dScgβ, 5'-CACCAGGGACGCAACACG-3' and 5'-TGCGGAAGACGCGCACAT-3', for dScgδ, 5'-ATTTCGCATAAACGACACCAA-3' and 5'-TCTGCAGACCGTGGAATCATC-3', and for rp49, 5'-ATGGCAACAAGCGCCAAACTG-3' and 5'-TCAGAAGCCCTTCTCCTTGAG-3'.

Immunohistochemistry of pupal retina

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 with diluted primary antibodies in PBST and 10% normal goat serum for 16 h at 4°C. Mouse anti-LacZ (1:500) (Promega) and rat anti-Elav (1:200) (DSHB) antibodies were used. Eyes were washed three times with PBST and then incubated with secondary antibodies labeled with either Alexa546 or Alexa488 (Invitrogen) at 25°C for 2h. After three washes in PBST, eyes were mounted in mounting medium (Bio-Rad) and analyzed by confocal laser scanning microscopy with a Zeiss LSM510.

Results

Drosophila and Mammalian Sarcoglycans

Since it is well known that mammalian Sarcoglycans form functional
hetero-tetramer [13,20], we examined the in vitro binding ability between dScgα and dScgβ, dScgα and dScgδ, or dScgβ and dScgδ by exploiting the pETDuet-1 co-expression system of two target genes in E. coli (Figure 1A). In this experiment, the specific binding between His tag and Ni agarose was utilized for precipitation, and co-precipitation of the counterpart protein containing Flag tag was analyzed by Western immunoblotting.

As shown in Figure 2 with input sample treated with anti-His and anti-Flag antibodies (Figure 2B, lanes i-iii and vii-ix), we confirmed the presence of all the target proteins whose cDNAs were carried by the distinct pETDuet-1 vectors in the supernatant after sonication of E. coli and centrifugation of protein extracts. Both His-dScgα and Flag-dScgα were detected at 52kDa by Western blotting (Figure 2B-I, II and IV), consistent with the expected molecular weight of dScgα 50.7kDa according to ExPASy (http://us.expasy.org/tools/pi_tool.html). The apparent molecular weights of His-dScgδ and Lamin were 41kDa and 72 kDa (Figure 2B-II, III and IV), respectively. These are also in accordance with the calculated molecular weights of dScgδ and Lamin.
molecular weights, 42.6kDa for dScgδ and 71.3kDa for Lamin. On the other hand, we found slightly larger apparent molecular weights of Flag-dScgβ (Figure 2B-I and II) and His-dScgβ (Figure 6B-II) at 48.0 kDa than the estimated molecular weight of dScgβ of 38.1kDa. The slightly slower migration of dScgβ on SDS-PAGE is consistent with our previous report [25]. Exogenously over-expressed dScgβ with Flag tag in flies also exhibited a molecular weight of 48.0 kDa [25]. Since both Flag tagged and His tagged dScgβ showed slower migration on SDS-PAGE, this behavior of dScgβ is likely due to intrinsic properties of the dScgβ amino acid sequence. 

We also ensured that His-tagged target proteins, His-dScgα and His-dScgδ, bind to Ni agarose gels as detected in the elution samples with anti-His antibodies (Figure 2B, lanes iv and vii). Flag-tagged dScgβ was detected by anti-Flag antibodies as co-precipitation products with His-tagged dScgα, indicating binding between dScgα and dScgβ (Figure 2B-I, lane xii). According to the quantification of the visible band by Lumivision Pro HSII image analyzer, approximately 12.4% of Flag-dScgβ in the input sample was eluted with His-dScgα. Similarly, we observed binding between His-dScgδ and Flag-dScgα, and between His-dScgδ and Flag-dScgβ (Figure 2B-II and III, lane xii). 12.5% of Flag-dScgα and 31.8% of Flag-dScgβ in the E. coli soluble lysate were eluted with His-dScgδ. On the other hand, Lamin, which shares similarities with Sarcoglycans as both are transmembrane proteins but which is not supposed to interact with any Sarcoglycans considering differences in sub-cellular localization, was undetectable on elution as expected (Figure 2B-IV, lane xii). These data suggest that the observed binding between dScgα and dScgβ, dScgα and dScgδ, and dScgβ and dScgδ was specific in each case (Figure 2B-I, II and III). Collectively, here we suggest that Drosophila Sarcoglycans can form heterologous complexes as with mammalian homologues, supporting the idea that so far identified Drosophila Sarcoglycans share characters with their corresponding mammalian counterparts. 

Alteration in EGFR signaling in knockdown flies for sarcoglycans

In a previous report, we demonstrated that knockdown of dscgβ (βi1) caused severe morphological aberrancy in the adult compound eyes accompanied by ectopic expression of rhomboid (rho) and consequently increased activation of EGFR signaling in pupal retinæ as evidenced by phosphorylation of ERK [19]. In the present study, initially in order to address the off-target effects which are frequently encountered with the RNAi technique, we have newly established and examined fly lines with dscgβ knockdown (βi2), whose target region for the inverted repeat sequence has no overlap with the previously tested construct (βi1) (Figure 1A). βi2 knockdown flies by GMR-GAL4 displayed a mild rough eye phenotype by SEM (Figure 3E). Both RT-PCR and Western blot analyses revealed less reduction of mRNA and protein levels of dScgβ in the βi2 knockdown flies than in βi1 knockdown flies (Figure 3N). βi2 knockdown flies showed reduction of mRNA level to 68% and reduction of protein level to 72% of those of the control flies, while βi1 knockdown flies exhibited 89% decrease in mRNA and 80% in protein (Figure 3N). The data could explain the weaker rough eye phenotype in βi2 than βi1 (Figure 3, panels D and E). However, although the extent of

Figure 4-Expression of rhomboid in 35-hour APF retinae.
Knockdown flies for any sarcoglycans showed ectopic expression of rhomboid in two unknown cells of each ommatidium. Red and green indicate anti-lacZ and anti-Elav signals, respectively. The white arrowheads indicate examples of cells ectopically expressing rhomboid. Although the level of the fluorescence differs in each sample, it does not simply reflect the differences in the expression level as this is mainly due to technical difficulties.

(A)GMR-GAL4/+;+;+/X63 (B)GMR-GAL4/+;+;UAS-IR-dscgβi1/X63 (C)GMR-GAL4/+;UAS-IR-dscgβi1;+;X63/+ (D)GMR-GAL4/+;UAS-IR-dscgδi1;+;X63/+ (E)GMR-GAL4/+;UAS-IR-dscgδi2;+;X63/+ (F)GMR-GAL4/+;UAS-IR-dscgδi2/X63 (G)GMR-GAL4/+;UAS-IR-dscgβi2/X63
Figure 5-Scanning electron micrographs of adult compound eyes. 
(A) GMR-GAL4/Y; UAS-IR-dscgα1/+; rhove-1/+ 
(B) GMR-GAL4/Y; UAS-IR-dscgα1/+; rhove-1/+ CyOrho−/−/+ 
(E) GMR-GAL4/Y; UAS-IR-dscgα1/+; rhove-1/+ 
Figure 6. In vitro binding between Drosophila Sarcoglycans and EGFR. 
(A) Diagrams of the obtained constructs for the production of recombinant proteins. The numbers written below the rectangular bars represent the amino acid numbers counted from the N-termini of the respective proteins. (B) Western blots of both Input and Elution samples with anti-His or anti-Flag antibodies are shown. Binding between the extracellular soluble form of Drosophila EGFR (Egfr 1-559) and variants of Sarcoglycans was examined. 5 mg of total protein in the E.coli extract was applied for a 100 μl volume of the Ni-NTA agarose gel and eluted with 100 μl buffer. 20 μl of each sample was loaded for the SDS-PAGE. While no binding of Egfr1-559 with dScgα was seen (I-lane 12), dScgβ and dScgδ demonstrated binding to Egfr1-559 (II and IV-lane 12). Furthermore, neither dScgβ nor dScgδ, lacking any EGF-like consensus sequence, showed binding to Egfr1-559 (III and V-lane 12), suggesting that the binding between dScgβ and Egfr1-559, dScgδ and Egfr1-559 is mediated by the EGF-like consensus sequence in the C-terminus regions of dscgβ and dscgδ.

Figure 7. Expression of Spitz and Argos in 35-hour APF pupal retinae. 
Red signals indicate expression of spitz (left panels) or argos (right panels), visualized by anti-lacZ antibody staining, whereas green signals indicates anti-Elav signals in photoreceptor cells. The white arrowheads indicate examples of the two extra cells per each ommatidium, showing expression of spitz (left panels) or argos (right panels) in the control (upper panels) or dscgβ knockdown (lower panels) flies. (left, upper) GMR-GAL4/+; spik05808/+; (left, lower) GMR-GAL4/+; spik05808/UAS-IR-dscgβi1; strain72)/+ (right, upper) GMR-GAL4/+; arg05845/+; (right, lower) GMR-GAL4/+; arg05845/UAS-IR-dscgβi1; strain72)/+
roughness in the adult compound eyes examined by SEM apparently differed between βi and βi2 knockdown flies, immunostaining data of 35 hour After Puparium Formation (APF) pupal retinas showed similar molecular alteration in terms of ectopic expression of rho in both βi and βi2 knockdown flies (Figure 4B and C). These phenotypic similarities of βi and βi2 knockdown flies ruled out off-target effects.

We also investigated knockdown effects of dscgα and dscgδ with GMR-GAL4 in order to determine whether the above-described phenotypes were induced by functional disorder of dscgβ itself or by the defects in the stability of the whole Sarcoglycan complex provoked by a single Sarcoglycan family protein depletion. Also here, two independent fly lines, whose target regions for inverted repeat constructs were not overlapping, have been established and tested to take into account the off-target problem (Figure 1A). RT-PCR data showed 79% reduction of mRNA level in αi, and 61%, 81%, and 69%, respectively in αi, δi1 and δi2 knockdown flies compared to control flies. Although knockdown flies carrying one copy of distinct transgenes (αi, αi2, δi and δi2) showed no obvious rough eye phenotype (Figure 3H-I), an enhanced rough eye phenotype was observed in the knockdown flies with two copies of each transgene (Figure 3B, C, F and G). In addition, synergistic enhancement was also evident in the double knockdown flies carrying inverted repeat sequences of two different sarcoglycans (Figure 3H-M). All the combinations of sarcoglycans, dscgα-dscgβ, dscgβ-dscgδ and dscgα-dscgδ exhibited an enhanced rough eye phenotype. Furthermore, we detected ectopic expression of rhomboid gene in the 35-hour APF pupal retinae of both dscgα and dscgδ knockdown flies as well as in dscgβ knockdown flies (Figure 4B-G). In addition, half dose reduction of the rho-1 gene significantly suppressed the rough eye phenotype induced by knockdown of dscgα or dscgδ (Figure 5), as observed with the dscgβ knockdown [19]. These results suggest that all three Sarcoglycans, dscgα, dscgβ and dscgδ are necessary for proper level of EGFR signaling to form normal compound eyes in adults.

Interactive mechanism of the Sarcoglycan complex and EGFR signaling

Although we indicated that Sarcoglycan complex could negatively regulate EGFR signaling in a physiological state [19], we still had no idea of how Sarcoglycan complex affects activation or inactivation of EGFR signaling. In order to address whether it is conducted by direct interaction with EGFR signaling molecules or via an indirect pathway, we performed an immunohistochemical expression pattern analysis of the major components in the EGFR signaling pathway like rho (Figure 4). In pupal retinae, only rhomboid showed different expression patterns between control and dscgβ knockdown flies (βi) (Figure 4). Since Rho is known as a rate-limiting protease of EGFR signaling and once EGFR signaling is activated, rho expression is further induced due to positive feedback regulation [26], Sarcoglycan complexes may regulate an upstream factor for Rhomboid. We also focused on the intriguing finding that dscgβ and dscgδ retain EGFR-like consensus sequences in their C-terminus regions, and these sequences are well conserved between Drosophila and mammals (Figure 1B). Therefore, dscgβ and dscgδ might directly interact with EGFR via the EGF-like consensus sequences and thus negatively regulate EGFR signaling.

In order to assess this hypothesis, we investigated binding between Drosophila EGFR (CG10079) and dscgβ or dscgδ in vitro (Figure 6). The soluble version of the human EGFR extracellular domain, comprising alpha human EGFR ligands such as human EGFR or TGFRa [27], showed strong binding affinity with well-known EGFR ligands such as human EGFR or TGFRa [27]. Therefore, in vitro binding assays, we generated a truncated and soluble form of Drosophila EGFR (Egfr1,559) composed of amino acid residues 1-559, which correspond to the human EGFR extracellular domain (Figure 6A).

On Western blot analysis, Egfr1,559 appeared at 63kDa, consistent with the estimated molecular weight 62.9kDa (Figure 6B). While no band was detectable by anti-Flag antibody in the elution sample of the binding assay between dscgα and Egfr1,559 (Figure 6B-I, lane xii), Flag tagged Egfr1,559 bands were detected as co-precipitated products with His tagged full lengths of dscgβ or dscgδ (Figure 6B-II and IV, lane xii), 5.3% of Flag-dscgβ and 22.3% of Flag-dscgδ in the Input extracts were eluted. For further analysis, we tested the binding between Egfr1,559 and truncated forms of dscgβ or dscgδ lacking EGF-like consensus sequences, namely dscgβΔEGF-like and dscgδΔEGF-like. Neither was detectable in the co-precipitated fraction with Egfr1,559 (Figure 6B-III and V, lane xii), indicating that dscgβ and dscgδ directly associate with the EGFR extracellular domain via their EGF-like consensus sequences. Taking into account the finding that knockdown of all three sarcoglycans in each case caused alteration in rhomboid expression in pupal retinae (Figure 4), we conclude that Sarcoglycans work as a whole complex in negative regulation of EGFR signaling through direct association of the EGFR consensus sequences in dscgβ and dscgδ with the extracellular domain of EGFR in Drosophila.

Argos and spitz expression in the pupal retinae

Both Spitz and Argos are known as regulatory proteins of EGFR signaling in Drosophila. While Spitz is a positively acting ligand, Argos acts in negative regulation by interrupting the association of Spitz with EGFR [27].

Interestingly, we detected both Argos and Spitz in the two extra cells per each ommatidium, which apparently showed ectopic expression of rhomboid in dscgβ knockdown flies [19] (Figure 4), both in the control flies and in the knockdown flies (Figure 7). Notably for Spitz, expression was detected only in these two cells but not in photoreceptor cells. Knockdown of Sarcoglycans may therefore trigger secretion of an activated form of Spitz in these two cells presumably as a consequence of primary defects in the proper inactivation of EGFR. This could abnormally accelerate over-activation of EGFR signaling by positive-feedback mechanism.

Discussion

In recent years, there are increasing number of the reports suggesting the cross talk between DGC and EGFR. For instance, genetic modifier screening using the visible wing vein phenotype of dystrophin and dystroglycan RNAi flies identified two genes, argos and kekkon-1 which both encode negative regulators of EGFR signaling [27-30].
Both dystrophin and dystroglycan mutants suppressed the posterior crossvein defective phenotype [30]. Moreover, EGFR signaling has been reported to regulate expression of dystroglycan in Drosophila oocytes [31]. We have also proposed in a previous report that Dscgβ interacts with EGFR signaling in Drosophila [19]. The data suggested a close relationship between DGC and EGFR signaling in a variety of tissues. However, the molecular mechanisms have remained elusive. Our present in vitro binding analysis of Drosophila Sarcoglycans and EGFR provides evidence of the linking mechanism.

EGFR signaling needs to be controlled precisely throughout animal development [32]. Especially, it is well known that the control of the proper level of EGFR signaling is vital for eye development in Drosophila since either hyperactive or hypoactive EGFR signaling causes abnormalities [33]. Hence, in order to adjust levels strictly, EGFR signaling receives complex modulation by multiple regulatory mechanisms, which must be coordinated [33,34]. In fact, there are a variety of ligands with distinct properties and interactions exist between EGFR signaling and other signaling pathways, such as the Notch pathway, Insulin receptor/Target of rapamycin kinase pathways, and the Frizzled/PCR pathway [35-37]. Sarcoglycans dependent negative regulation is also likely from the data presented in this report. Sarcoglycans appear to work for inactivation of EGFR via direct association in pupal retinae (Figures 4 and 6). Dscgβ and Dscgδ, both of which retain EGF-like consensus sequences in their C-termini, showed binding to EGFR binding region of EGFR (Figures 6B-II and IV). In contrast, Dscgα, which carries no EGF-like consensus sequence, did not display binding (Figure 6B-II). Furthermore, the truncated forms of Dscgβ and Dscgδ lacking the EGF-like consensus lost the binding ability (Figure 6B-III and V). These EGF-like consensus sequence in Dscgβ and Dscgδ may competitively inhibit Spitz binding to EGFR. Therefore, we suggest that the negative regulation of EGFR signaling is, at least in part, controlled by direct association of EGFR with Dscgβ and Dscgδ.

It has been reported that ectopic expression of dystroglycan is induced in both egfr mutant clone cells and in null mutant clone cells of Ras in posterior follicle cells, whereas, mis-expression of the constitutively active form of EGFR caused down-regulation of dystroglycan expression in anterior follicle cells. Both observations are consistent in suggesting that EGFR signaling regulates expression of dystroglycan during oogenesis [31]. Dystroglycan plays a role in determining cellular polarity and in axis formation during oogenesis [31,38]. An adequate level of Dystroglycan expression controlled by EGFR signaling and a gradient in Dystroglycan expression could be required to establish the anterior-posterior axis in oogenesis [31]. Feedback regulation from the DGC side to cause up-regulated EGFR signaling might contribute to maintaining this gradient of Dystroglycan expression. In the eyes, the breakdown of the DGC induced by knockdown of sarcoglycans might stimulate the feedback effect and lead to over-activation of EGFR signaling as observed in this study.

Interestingly, several published studies have shown that no or reduced expression of α-dystroglycan or β-dystroglycan frequently occurs in human cancers [38-40]. Alteration of dystroglycan expression is unlikely to be the primary reason for tumorigenesis as reduction of Dystroglycan levels in normal cells does not induce tumor formation [39,41] and none of the DGC related muscular dystrophies is reported to be linked with remarkable increase in tumor occurrence in human patients. However, Dystroglycan at least appears to act as a tumor suppressor since re-expression of dystroglycan in breast cancer cells, in which dystroglycan expression was originally decreased, significantly reduced tumor size [42]. Furthermore, it is well known that EGFR signaling is over-activated and/or miss-regulated in many solid tumors [43]. Abnormal activation of EGFR signaling triggers processes that promote tumor cell proliferation, migration, and angiogenesis. It is of interest that, also with respect to tumor genesis, the DGC and EGFR signaling present reciprocal actions. Further elucidation of interplay between DGC and EGFR signaling during tumor progression might shed light on novel strategies for better management of cancer therapy by exploiting DGC activity against EGFR signaling [43].

Conclusions

In vitro data in combination with immunohistochemical data for sarcoglycan knockout fly retinae, we propose that Sarcoglycan complex formation is required for eye development. Notably, the direct binding of β-Sarcoglycan and δ-Sarcoglycan to EGFR might be critical for the proper regulation of EGFR signaling during eye development in Drosophila.

Competing interests

The authors declare that they have no competing interests.

Authors’ contribution

RH participated in designing experiments, carried on in vitro binding assays and immunostaining experiments. MY carried out establishment of transgenic flies. HY carried out genetic interaction analyses. RH, HY and MY participated in writing the manuscript.

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