Drosophila DOCK Family Protein Sponge Regulates the JNK Pathway during Thorax Development

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ABSTRACT. The dedicator of cytokinesis (DOCK) family proteins that are conserved in a wide variety of species are known as DOCK1–DOCK11 in mammals. The Sponge (Spg) is a Drosophila counterpart to the mammalian DOCK3. Specific knockdown of spg by pannier-GAL4 or apterous-GAL4 driver in wing discs induced split thorax phenotype in adults. Reduction of the Drosophila c-Jun N-terminal kinase (JNK), basket (bsk) gene dose enhanced the spg knockdown-induced phenotype. Conversely, overexpression of bsk suppressed the split thorax phenotype. Monitoring JNK activity in the wing imaginal discs by immunostaining with anti-phosphorylated JNK (anti-pJNK) antibody together with examination of lacZ expression in a puckered-lacZ enhancer trap line revealed the strong reduction of the JNK activity in the spg knockdown clones. This was further confirmed by Western immunoblot analysis of extracts from wing discs of spg knockdown fly with anti-pJNK antibody. Furthermore, the Duolink in situ Proximity Ligation Assay method detected interaction signals between Spg and Rac1 in the wing discs. Taken together, these results indicate Spg positively regulates JNK pathway that is required for thorax development and the regulation is mediated by interaction with Rac1.

Key words: Sponge, DOCK, JNK, Rac1, Drosophila

Introduction

Rho GTPases and GTPase regulatory proteins, such as GTPase-activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and guanine nucleotide dissociation inhibitors, (GDIs) are important signaling molecules that involve various developmental processes including cell migration, phagocytosis and myoblast fusion (Meller et al., 2005; Bos et al., 2007; Cote and Vuori, 2007; Garcia-Mata and Burridge, 2007). The dedicator of cytokinesis (DOCK) family proteins are known as GEF proteins and are conserved among a wide variety of species from Monosiga brevicollis to human (Meller et al., 2005; Cote and Vuori, 2007).

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In mammals, eleven DOCK family proteins, DOCK1 (DOCK180) to DOCK11 have been identified (Meller et al., 2005; Cote and Vuori, 2007). Based on their amino acid sequence homology, DOCK family proteins can be further classified into four subfamilies DOCK-A, -B, -C and -D (Lu et al., 2005; Meller et al., 2005; Cote and Vuori, 2007). Mammalian DOCK1, 2 and 5 belong to DOCK-A; DOCK3 and 4 belong to DOCK-B; DOCK6, 7 and 8 belong to DOCK-C; DOCK9, 10 and 11 belong to DOCK-D. DOCK1, 2, 3, 4 and 5 proteins commonly contain the N-terminal Src-homology-3 (SH3) domain, DOCK homology regions (DHR) 1 and 2, and the C-terminal proline-rich region. The SH3 domain binds to proline-rich regions of the protein ELMO, the DHR1 domain binds to phosphatidylinositol-3,4,5-triphosphate [PtdIns(3,4,5)P3] and the DHR2 domain functions in the catalytic activity of GEFs (Brugnera et al., 2002; Cote and Vuori, 2002, 2007; Grimsley et al., 2004). ELMO was originally identified in Caenorhabditis elegans as an upstream regulator of Rac in apoptotic cell engulfment and cell migration (Wu and Horvitz, 1998; Gumienny et al., 2001; Zhou et al., 2001). Current studies with mammalian ELMO1 showed that the
DOCK1–ELMO complex is required for Rac-mediated cell migration and phagocytosis (Brugnera et al., 2002; Katoh and Negishi, 2003; deBakker et al., 2004; Grimsley et al., 2004; Lu et al., 2004). Other DOCK-A family proteins also exchange the nucleotide of the Rac protein. However, it is reported that DOCK4, which belongs to the DOCK-B subfamily, can also exchange the nucleotide of the Rap protein (Yajnik et al., 2003). Many studies have shown that DOCK1 is involved in neurite outgrowth and myoblast fusion and DOCK2 is involved in lymphocyte migration in response to chemokines and differentiation of lymphocytes (Katoh et al., 2000; Balagopalan et al., 2006). In addition, DOCK3 is reported to be involved in neuronal differentiation (Namekata et al., 2012).

Four DOCK family proteins have been identified in Drosophila. Myoblast city (Mbc), Sponge (Spg)/CG31048, Zizimin-related (Zir)/CG11376 and Zizimin (Ziz)/CG42533 belong to DOCK-A, -B, -C and -D subfamilies, respectively (Rushoton et al., 1995; Erickson et al., 1997; Meller et al., 2005; Biersmith et al., 2011; Sampson et al., 2012). It is reported that Mbc, a DOCK-A subfamily, appears to have a function similar to that of mammalian DOCK1 and is involved in myoblast fusion (Erickson et al., 1997; Bour et al., 2000). Spg is a member of the DOCK-B subfamily in Drosophila. Recent studies have shown that Spg is involved in embryonic central nervous system (CNS) development (Biersmith et al., 2011) and differentiation of R7 photoreceptor cells (Eguchi et al., 2013). Zir is a member of the DOCK-C subfamily in Drosophila involving a proper cellular immune response (Sampson et al., 2012). However, Ziz is poorly characterized.

In the present study, we focused on spg and examined the effect of knockdown of spg in the notum region of wing disc by a pannier (pnr)-GAL4 driver. Knockdown of spg induced a split thorax phenotype similar to those of mutants in genes involved in the c-Jun N-terminal kinase (JNK) cascade. The JNK cascade is an intracellular signaling pathway in which the stress-activated kinases Jun N-terminal kinase (JNKK) and JNK play essential roles (Martin-Blanco et al., 2000). In Drosophila, JNKK and JNK homologs are encoded by the genes hemipterous (hep) and basket (bsk), respectively (Sluss et al., 1996; Agnes et al., 1999). Mutations in each of these genes show an embryonic dorsal-open and adult split thorax phenotype, as a consequence of the lack of elongation of cells of the lateral epidermis (Glise et al., 1995). During closure, JNK signaling activity is modulated by the product of the gene puc (puc) that encodes a dual-specificity phosphatase (Martin-Blanco et al., 1998). The puc is expressed in the dorsal-most cells of the epidermis (“leading-edge” cells), and cell recognition at the dorsal midline is impaired in its absence (Martin-Blanco et al., 1998). It is also known that Rho family GTPases Rac1 and Cdc42 regulate the activity of JNK signal pathway in Drosophila (Omar et al., 1995; Glise and Noselli, 1997). Knockdown of Mbc and ELMO by pnr-GAL4 induces split thorax phenotype, and upstream of Mbc receptor pvr activates JNK pathway (Ishimaru et al., 2004). Although DOCK3, a mammalian homologue of Spg is reported to activate Rac1 (Namekata et al., 2004), its biological significance is still unclear due to a lack of appropriate in vivo studies. Extensive genetical and cell biological analyses in the present study demonstrated that Spg positively regulates JNK pathway that is required for thorax development and the regulation is mediated by interaction with Rac1.

**Materials and Methods**

**Fly stocks**

Fly stocks were cultured at 25°C on standard food. Canton S was used as the wild type strain. The transgenic fly lines carrying UAS-spgr1447-1632 and UAS-spgr900-1092 were described earlier (Eguchi et al., 2013). puc900/TM6C lines were kindly provided by Dr. T. Adachi-Yamada. pnr-GAL4, ap-GAL4, bsk1/CyO, bsk2/CyO, UAS-Bsk, UAS-GFP, UAS-GFPIR used in this study were obtained from the Kyoto Drosophila Genetic Resource Center, the Bloomington Drosophila Stock Center or Vienna Drosophila Genetic Resource Center.

**Purification of anti-Spg IgG**

Guinea pig anti-Spg IgG was purified from anti-Spg serum (Eguchi et al., 2013) by using Protein A Mag Sepharose TM Xtra (GE Healthcare) according to the supplier’s recommendations. The purified IgG was diluted with PBS to 2 μg/μL in use.

**Western immunoblot analysis**

Protein extracts were prepared from the wing imaginal discs. After dissection, the discs were dissolved in sample buffer (62.5 mM Tris-HCl pH 6.8, 2.5% SDS, 0.002% Bromophenol Blue, 0.7135 M β-mercaptoethanol, 10% glycerol) containing Protease Inhibitor Cocktail (Nacalai tesque) and Phosphatase Inhibitor Cocktail (Sigma) at 1:4,000 for 16 hours at 4°C. The bound antibodies were detected with the peroxidase-conjugated anti-mouse IgG or anti-guinea pig IgG and the ECL system (GE Healthcare) according to the manufacturer’s recommendations. The images were analyzed with AE-9300H Ez-Capture MG image analyzer (ATTO).
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**Flip-out experiments**

RNAi clones in eye discs were generated with a flip-out system (Sun and Tower, 1999). Female flies with hs-flp; Act5C-FRT y FRT>GAL4, UAS-GFP were crossed with male flies with UAS-spgIR and clones were identified by the presence of green fluorescent protein (GFP) expressed under control of the Act5C promoter. Flip-out was induced by heat shock (30 min at 37°C) at 24–48 hours after egg laying. In order to express both spg dsRNA and Bsk, female flies with hs-flp; Act5C>FRT y FRT>GAL4, UAS-GFP; UAS-spgIR were crossed with male flies with UAS-Bsk.

**Scanning electron microscopy**

Adult flies were anesthetized, mounted on stages and observed with a scanning electron microscope in low vacuum mode (VE-7800, Keyence Inc.). The thorax phenotype of at least five adult male flies (3 to 5 days old) of each line was examined in each experiment and the experiments were done in triplicate. No significant variation in thorax phenotype was observed among the five individuals.

**Immunohistochemistry**

Third instar larvae were dissected in *Drosophila* Ringer’s solution and the wing imaginal discs were fixed in 4% paraformaldehyde in PBS for 15 min at 25°C. After washing with PBST, the samples were blocked with PBST containing 10% (v/v) normal goat serum incubated at 4°C for 16 hours with mouse monoclonal anti-β-galactosidase antibody (DSHB) (1:500), guinea pig anti-Spg IgG (1:2,000), anti-pJNK G9 antibody (CST) (1:100), mouse monoclonal anti-Rac1 antibody (BD Transduction Laboratories) (1:500). After extensive washing with PBST, imaginal discs were incubated for 3 hours at 25°C with goat anti-mouse IgG conjugated with Alexa 488, goat anti-rabbit IgG conjugated with Alexa 594 or goat anti-guinea pig IgG conjugated with Alexa 488 or 594 (Invitrogen) (1:400). After extensive washing with PBST and PBS, samples were mounted in VECTASHIELD (Vector) and inspected with an Olympus FV-10i (Olympus) laser confocal microscopy.

**Proximity ligation assay**

Duolink in situ Proximity ligation assay (PLA) kits were purchased from Olink. Fixation of wing imaginal discs, blocking of nonspecific binding of antibody and immunostaining with anti-Spg and anti-Rac1 antibodies were done as described above. Subsequently, secondary antibodies conjugated with the PLA oligonucleotide probes were used: Duolink II PLA probe anti-mouse MINUS, and anti-guinea pig antibody (Jackson Immuno Research) that conjugated Duolink II Probmaker PLUS. Ligation of the connector oligonucleotides, rolling-circle amplification and detection of the amplified DNA products were done with Duolink II Detection Reagents Red (Olink Bioscience) according to the manufacturer’s instructions.

**Data analysis**

Quantification of intensity of pJNK signals or split thorax phenotype was carried out with six to nine different samples by using Meta Morph software (Molecular Devices) or Image J. For the statistical analysis, Microsoft Excel 2007 was used. P-values were calculated using Welch’s t-test and the error bars represent Standard Errors from Means.

**Results and Discussion**

**Localization of Spg protein in the wing imaginal discs**

It has been reported that Spg is expressed in cytoplasm of photoreceptor cells with highest expression in R7 cells in eye imaginal discs (Eguchi *et al.*, 2013). We examined localization pattern of Spg in wing imaginal discs of wild type Canton S by immunostaining with anti-Spg IgG (Eguchi *et al.*, 2013). Specificity of the antibody has been reported in our previous studies (Eguchi *et al.*, 2013). Spg signals are detected throughout the wing discs with relatively stronger signals in the body wall that becomes notum in the adults (Fig. 1A). The effects of Spg double stranded RNA (dsRNA) on the endogenous spg expression were examined by a flip-out experiment. Cells marked with GFP express spg dsRNA (Fig. 1D). The level of Spg signal was reduced in the RNAi clone area (Fig. 1C and E). Higher magnification image also reveals cytoplasmic localization of Spg in the body wall cells (Fig. 1C and E). These data reveal the effective knockdown of spg in the wing discs and also confirm again that the anti-Spg IgG is highly specific to the Spg protein.

**Knockdown of spg induced split thorax phenotype**

To investigate a role of spg in the thorax development, we examined the effect of the reduction of Spg protein in the wing imaginal discs using a combination of the GAL4-UAS targeted expression system and the RNAi method. We used pnr-GAL4 and apterous (ap)-GAL4 driver to induce expression of spg dsRNA. The pnr-GAL4 specifically expresses GAL4 in body wall of the wing disc and the ap-GAL4 expresses GAL4 in wider region including the wing pouch and the body wall.

Expression of spg dsRNA with pnr-GAL4 induced the split thorax phenotype (Fig. 2C-F) in compared with the split thorax phenotype level are shown in Fig. 1G. The +; +; pnr-GAL4/UAS-GFPIR flies show ratio of 0.027. The UAS-spgIR; +/+ flies show ratio of 0.062 that is a 2.3 fold increase. The +; +; pnr-GAL4/UAS-spgIR flies show ratio of 0.102 that is a 3.8 fold increase. These values show statistically significant
increase in split area of thorax of the spg knockdown flies (Fig. 2G). Expression of dsRNA targeted to both aa900-aa1092 and aa1447-aa1632 induced similar split thorax phenotype, although the split thorax phenotype was severer with flies carrying the UAS-\textit{spgIR}_{1447–1632} (Fig. 2C-F). The data exclude the possible off-target effect on the thorax phenotype.

Specific knockdown of \textit{spg} with \textit{ap}-GAL4 also induced the split thorax phenotype (Fig. 3C-F) together with blistering wing phenotype (data not shown). The quantified data of split thorax phenotype level, ratios of split area in notum area are shown in Fig. 3G. The +; \textit{ap}-GAL4/+; UAS-GFPIR/+ flies show ratio of 0.035. The \textit{UAS-spgIR}_{900–1092}/+; \textit{ap}-GAL4/+; + flies show ratio of 0.051 that corresponds to 1.5 fold increase and the +; \textit{ap}-GAL4/+; UAS-spgIR_{1447–1632}/+ flies show ratio of 0.068 that corresponds to 1.9 fold increase. Therefore, knockdown of \textit{spg} by the \textit{ap}-GAL4 driver also showed statistically significant increase in split area in the thorax (Fig. 3G). These data taken together suggest that \textit{spg} plays a role in thorax development.

\textit{The spg genetically interacts with the basket gene}

Thorax development requires the JNK signaling pathway (Agnes \textit{et al.}, 1999; Zeitlinger and Bohmann, 1999; Ishimaru \textit{et al.}, 2004). It is therefore known that reduction of \textit{Drosophila} JNK gene \textit{bsk} results in a split thorax phenotype similar to those observed with \textit{pnr}-GAL4>UAS-spgIR flies or \textit{ap}-GAL4>UAS-spgIR flies. Therefore we considered that knockdown of \textit{spg} may attenuate JNK signaling through genetic interactions. To explore this possibility, we firstly crossed the \textit{UAS-SpgIR}_{900–1092}/+; +; \textit{pnr}-GAL4/+ with flies overexpressing the wild type \textit{bsk} gene to examine any effects on the split thorax phenotype (Fig. 4). Significant enhancement of \textit{spg} knockdown-induced split thorax phenotype was observed by expression of GFP (Fig. 4E, F), although at the moment we do not know why the overexpression of GFP worsens the split thorax phenotype. In any event strong suppression of the split thorax phenotype induced by knockdown of \textit{spg} was observed in flies overexpressing the wild type \textit{bsk} (Fig. 4G, H). Overexpression of \textit{Bsk} alone exerted only a marginal effect on the thorax development (Fig. 4A, B). In contrast, genetic crossing of \textit{spg} knockdown flies, \textit{pnr}-GAL4>UAS-spgIR with \textit{bsk}\textsuperscript{1} resulted in enhancement of the split thorax phenotype (Fig. 4I, J). Crossing with the other allele, \textit{bsk}\textsuperscript{2} resulted in enhancement of the split thorax phenotype to the similar extent (Fig. 4K, L). These results indicate that the \textit{spg} genetically interacts with the \textit{bsk} gene and also suggest that the \textit{spg} positively regulates the JNK signaling pathway.
Fig. 2. Knockdown of spg by pnr-GAL4 induces split thorax phenotype. Scanning electron micrographs of adult notum. (A, B) pnr-GAL4/UAS-GFP1R. (C, D) UAS-spgIR<sub>900–1092</sub>+/++; pnr-GAL4/+. (E, F) pnr-GAL4/UAS-spgIR<sub>1447–1632</sub>. (B, D, F) Magnified images of A, C and E. The split area in the thorax is enclosed by white line. The flies were developed at 28°C. The bars indicate 100 μm. (G) The quantified data of the split thorax phenotype. Ratio of split area to the whole notum area enclosed by white broken line is shown. *, significant difference (Student’s t-test, P<0.05); ** P<0.01. Error bar indicates ±SEM. N=6
Fig. 3. Knockdown of spg by ap-GAL4 induces split thorax phenotype. Scanning electron micrographs of adult notum. (A, B) ap-GAL4/+; UAS-GFPIR/+. (C, D) UAS-spgIR<sub>900-1092</sub>/+; ap-GAL4/+; +. (E, F) +; ap-GAL4/+; UAS-spgIR<sub>1447-1632</sub>/+. (B, D, F) Magnified images of A, C and E. The split area in the thorax is enclosed by white line. The flies were developed at 28°C. The bars indicate 100 μm. (G) Quantified data of the split thorax phenotype. Ratio of split area in notum to the whole notum area enclosed by white broken line is shown. * and ** indicate significant differences (Student’s t-test, * P<0.05 and ** P<0.01). Error bar indicates ±SEM.
The observation that mutations in \( bsk \) enhance the \( Spg \) knockdown-induced split thorax phenotype suggests that expression and/or activation of \( bsk \) is reduced in the \( Spg \) knockdown flies. To further explore this possibility, we measured \( bsk \) activation using an enhancer trap line, \( pucE69 \), in which \( lacZ \) is inserted into the \( puc \) gene intron (Martín-Blanco et al., 1998; Adachi-Yamada, 2002) so that \( puc \) enhancer activity can be monitored with reference to \( lacZ \) expression. It is well known that the \( puc \) gene is highly expressed in \( bsk \)-activated cells (Martín-Blanco et al., 1998; Adachi-Yamada, 2002) and the \( puc \) enhancer trap line has been widely used to monitor \( bsk \) activity in vivo (Tateno et al., 2000; Adachi-Yamada, 2002; Igaki et al., 2002). The \( lacZ \) of the \( puc\)-LacZ enhancer trap line is exclusively expressed in the stalk region of wing imaginal discs (Fig. 5A) that is consistent with the reported expression pattern of the \( puc \) gene in the wing imaginal discs (Adachi-Yamada, 2002). We have carried out the flip-out experiments to produce somatic clones expressing \( spg \) dsRNA and monitored \( puc \) expression in the stalk region by immunostaining of the wing discs with anti-lacZ antibody. In the flip-out experiments, cells marked with GFP are expressing \( spg \) dsRNA (Fig. 6A, E). In the RNAi clone area, the level of the \( puc\)-lacZ signal is reduced (Fig. 5C, E, inset, F and H).

To more directly prove JNK activation in the \( Spg \) knockdown wing discs, we monitored the activated JNK by immunostaining of the wing discs with anti-phosphorylated-JNK (pJNK) antibody. The pJNK signals are detectable at ventral end in body wall of the wing disc. Cells marked with GFP are expressing \( spg \) dsRNA (Fig. 6A, E). In the RNAi clone area, the level of the pJNK sig-
naling is reduced (Fig. 6B, C and F, G, inset). These results, taken together revealed that knockdown of spg reduces pJNK signals in the wing imaginal disc. In addition, overexpression of the wild type Bsk induced the activated pJNK signals in spg knockdown area (Fig. 7). The results are consistent with the data shown in the Fig. 4G, H indicating that the split thorax phenotype induced by spg knockdown was effectively suppressed by overexpressing the wild type Bsk. Moreover, we examined pJNK level by Western immunoblotting analysis with the extracts from third instar larval wing discs (Fig. 6I). We prepared extracts from wing discs of +; ap-GAL4/+; UAS-GFPIR/+ flies for control, and those of the +; ap-GAL4/+; UAS-spgIR1447–1632/+ flies for spg knockdown. In these experiments we used ap-GAL4 driver that expresses GAL4 in a wider region in the wing pouch including body wall. The Western blots detected a single pJNK band and a control α-tubulin band. The quantified data of the pJNK band are shown as ratio of pJNK level over α-tubulin level. We adjusted control ratio to be 1.0. In the wing discs of the spg knockdown flies, pJNK level was reduced to 12%. These results taken together indicate the Spg involvement in activation of Bsk during thorax and wing development.

Spg interacts with Rac1 in the wing disc

It is reported that DOCK3 and DOCK4 activate Rac1 in mammals (Namekata et al., 2004) and only DOCK4 activates Rap1 specifically (Yajnik et al., 2003). Spg is a Drosophila counterpart of DOCK3. Moreover, Rho a family GTPases, Rac1 and Cdc42 regulate the activity of JNK signal pathway (Omar et al., 1995, Glise and Noselli, 1997). Since direct interaction between Spg and Rac1 has not been demonstrated in vivo, we examined whether the both proteins can interact each other in the wing disc cells. For this purpose, we used the Duolink in situ PLA method (Olink Bioscience). This method has been widely used to monitor endogenous protein–protein interactions at subcellular levels (Laane et al., 2009; Tuomi et al., 2009; Yamazaki et al., 2009; Leuchowius, 2010; Eguchi et al., 2013).

In order to prove specificity of the protein–protein interaction, we combined the flip-out experiments with the PLA method. The interaction signals between Spg and Rac1 are detectable in the wing imaginal disc (Fig. 8B, E). Cells marked with GFP are expressing spg dsRNA (Fig. 8A, D). In the RNAi clone area, level of the PLA signals is reduced (Fig. 8B, C and E, F). These results suggest that Spg is involved in JNK activation and thorax development via...
Many studies have shown that the mammalian DOCK family regulates a variety of developmental processes, including cell migration, phagocytosis and myoblast fusion by activating GEFs (Brugnera et al., 2002; Cote and Vuori, 2002; Katoh and Negishi, 2003; deBakker et al., 2004; Grimsley et al., 2004; Lu et al., 2004). In Drosophila, Mbc regulates myoblast fusion and development of embryonic CNS by binding to ELMO protein to activate Rac1 that plays a role in regulation of the actin cytoskeleton. Spg plays a role in embryonic CNS development via binding to the ELMO protein (Biersmith et al., 2011). The phenotype of the Rac1 mutant is similar to those of the Mbc and spg mutants in embryonic CNS development (Obara et al., 2007), although spg is not involved in embryonic myoblast fusion (Biersmith et al., 2011).

Mutations in the JNK cascade genes show an embryonic dorsal-open and adult split thorax phenotype, as a consequence of the lack of elongation of cells of the lateral epidermis (Glise et al., 1995). In mammals, Rac1 interacts with both DOCK3 and DOCK4 proteins. Knockdown of Mbc and ELMO induces split thorax phenotype, and in the upstream of Mbc the receptor Pvr activates JNK pathway (Ishimaru et al., 2004). Similarly, in the present study, we showed that knockdown of spg induces split thorax phenotype and activates the JNK cascade. It is therefore likely that Spg somehow cooperates with Mbc to activate JNK cascade via Rac1 activation during Drosophila thorax development.
Fig. 7. Overexpression of Bsk increases phosphorylated JNK signals in *spg* knockdown area of the wing imaginal discs. (A–D) Flip-out experiments. (A) Cells expressing both *spg* dsRNA and wild type Bsk are marked with GFP. (B) Enlarged image of the region enclosed by a white box in panel A. GFP positive region is enclosed by a dotted line. (C) Immunostaining of the wing disc with anti-pJNK IgG. (D) Enlarged image of the region enclosed by a white box in panel C. GFP positive region in panel B is enclosed by a dotted line. The bars indicate 100 μm. a, anterior; p, posterior.

Fig. 8. Spg interacts with Rac1 in the wing imaginal disc. Duolink *in situ* PLA combined with the Flip-out experiment. PLA signals indicate Spg-Rac1 interaction. (A–F) Body wall regions of the wing discs are shown. (A, D) Cells expressing *spg* dsRNA are marked with GFP. (B, E) PLA signals. (C, F) Merged image of GFP (green) and PLA (red) signals. The inset in panel C shows enlarged image of the region enclosed by a white box. PLA signals are detected in the cytoplasm. In the GFP positive area enclosed with the white broken line in panels B and E, PLA signals are reduced. The bars indicate 30 μm. a, anterior; p, posterior.
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