βPS-Integrin acts downstream of Innexin 2 in modulating stretched cell morphogenesis in the Drosophila ovary

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Abstract

During oogenesis, a group of specialized follicle cells, known as stretched cells (StCs), flatten drastically from cuboidal to squamous shape. While morphogenesis of epithelia is critical for organogenesis, genes and signaling pathways involved in this process remain to be revealed. In addition to formation of gap junctions for intercellular exchange of small molecules, gap junction proteins form channels or act as adaptor proteins to regulate various cellular behaviors. In invertebrates, gap junction proteins are Innexins. Knockdown of Innexin 2 but not other Innexins expressed in follicle cells attenuates StC morphogenesis. Interestingly, blocking of gap junctions with an inhibitor carbenoxolone does not affect StC morphogenesis, suggesting that Innexin 2 might control StCs flattening in a gap-junction-independent manner. An excessive level of βPS-Integrin encoded by myospheroid is detected in Innexin 2 mutant cells specifically during StC morphogenesis. Simultaneous knockdown of Innexin 2 and myospheroid partially rescues the morphogenetic defect resulted from Innexin 2 knockdown. Furthermore, reduction of βPS-Integrin is sufficient to induce early StCs flattening. Taken together, our data suggest that βPS-Integrin acts downstream of Innexin 2 in modulating StCs morphogenesis.

Keywords: Innexin; integrin; microtubule; oogenesis; morphogenesis; stretched cells; follicle cells

Introduction

Epithelial morphogenesis is key to construct developing tissues and organs. During morphogenesis, communication through signal transduction or direct intercellular connection is required for the coordination of cell shape change and rearrangement. Gap junctions form direct intercellular connection, which is an effective way for synchronizing cells such as neurons and cardiomyocytes (Rohr et al. 2004; Sohl et al. 2005). In addition, gap junction proteins are essential for pattern formation during development (Levin 2007). However, the underlying mechanisms of morphogenesis regulated by gap junction proteins remain to be investigated.

Gap junctions are composed of juxtaposed channels on adjacent cells. These juxtaposed channels are formed by Connexins (Cxs) in vertebrates and Innexins (Inxs) in invertebrates. Six Cxs constitute a Connexon; and two Connexons form a gap junction (Goodenough et al. 1996; Revel et al. 1985). Inxs, on the other hand, may form hexadecameric gap junctions (Oshima et al. 2016). Gap junctions allow connected cells to transmit small molecules and metabolites such as calcium, inositol phosphates, and cyclic nucleotides (Nielsen et al. 2012). In addition, gap junction proteins interact with other proteins to regulate cell adhesion or cell polarity independent of their gap junction properties (Bauer et al. 2004; Elias et al. 2007; Giuliani et al. 2013; Ghezali et al. 2018).

Thus, it is important to unravel functions or mechanisms of gap junction proteins in modulating various biological processes.

In Drosophila, there are eight Inx genes playing diverse roles in different tissues (Bauer et al. 2005; Phelan 2005). Many finding suggest that Inxs have critical functions in the neuronal circuit of the nervous system, including Inx6, Inx7, and Shaking B (ShakB or Inx8) (Phelan et al. 1996; Sun and Wyman 1996; Phelan et al. 1998; Wu et al. 2011). Furthermore, mutation of optic ganglion reduced (ogre or Inx1) causes smaller optic lobes due to reduction of postembryonic neuroblasts (Lipshitz and Kankel 1985). Gap junctions composed of Inx1 and Inx2 in glial cells of the blood-brain barrier coordinate calcium oscillations in response to nutritional signals and control secretion of insulin (Holcroft et al. 2013; Speder and Brand 2014). In addition, gap junction proteins are important for epithelial morphogenesis. Inx2 and Inx3 regulate epithelial organization and polarity in the embryonic epidermis (Lehmann et al. 2006). Inx3 is required for dorsal closure, a morphogenetic event during mid-embryogenesis (Giuliani et al. 2013). Inx2 is a target gene of the Wingless pathway mediating morphogenetic movements during gut organogenesis (Bauer et al. 2002). In the embryonic epithelia, Inx2 directly interacts with Drosophila E-Cadherin (DE-cad) and Armadillo (Arm), demonstrating that Inx2 regulates cell polarity through protein–protein interaction with components of cellular junctions (Bauer et al. 2004).
To investigate functions of gap junction proteins, the Drosophila ovary provides a convenient model (Rubin and Huynh 2015). The Drosophila ovary is composed of 15–20 ovarioles containing egg chambers at different stages. Based on morphological characters and cell cycle status, oogenesis is categorized into 14 stages (Jia et al. 2016). The germarium located at the anterior tip of the ovariole contains both germline and follicle stem cells (FSCs), which divide and generate egg chambers with 16-cell germline cysts enwrapped by a monolayer of follicular epithelium. Egg chambers move posteriorly and develop into eggs (Bastock and St John 2008). Prior to stage 6, follicle cells undergo mitosis and produce about 650 cells. At stage 7, the Notch pathway drives the switch from mitosis to endocycle (Deng et al. 2001). From stage 9 to 10, follicle cells reorganize through a series of migratory and morphogenetic behaviors. Six to eight anterior-most follicle cells are specified into border cells and migrate with the anterior polar cells toward the border between nurse cells and the oocyte. About 50 anterior follicle cells are specified into stretched cells (StCs) and flatten drastically to cover nurse cells. At the same time, posterior follicle cells form a layer of columnar epithelium surrounding the oocyte (Wu et al. 2008).

Several genes and signaling pathways that regulate morphogenesis of StCs have been identified. Results from a quantitative morphometric analysis suggest that morphogenesis of StCs may be passively caused by enlargement of germline cells (Kolahi et al. 2009). Another study suggests that the Transforming Growth Factor β (TGF-β) pathway promotes StC flattening via activating the Notch pathway and remodeling of the adherens junctions (Brigaud et al. 2015), suggesting that StC morphogenesis is active. Hindsight (Hnt), a Zinc-finger containing transcription factor, modulates StC morphogenesis by down-regulating adhesion molecules, such as Arm, DE-cad, DN-cad, and Fasciclin 3 (Fas3) (Melani et al. 2008). A Ser/Thr kinase Tao modulates StC flattening by promoting endocytosis of Fasciclin 2 (Fas2) at the lateral membrane prior to StC morphogenesis (Gomez et al. 2012). In addition, adhesion molecules such as integrins are critical for cell morphogenesis (Daley and Yamada 2013). Integrins are β heterodimeric cell surface receptors connecting the extracellular matrix to the cytoskeleton (Bulgakova et al. 2012). In Drosophila, there are two β subunits, βPS and βn, and five α subunits of integrins. βPS encoded by myospheroid (mys) is the only β subunit detected in the oocyte (Fernandez-Minan et al. 2008). However, roles of integrins in StC morphogenesis have not been carefully examined (Chlasta et al. 2017).

Gap junction proteins have been shown to play various roles during oogenesis. Zero population growth (zpg or Inx4) is required for the differentiation of both male and female germlines (Gilboa et al. 2003; Smendziuk et al. 2015). Microinjection of anti-Inx2 antibodies into the oocyte stops oogenesis (Bohrmann and Zimmermann 2008). A mutant allele of Inx2 leads to aberrant cyst and egg chamber formation (Mukai et al. 2011). Inx2 is also required for border cell induction through modulating calcium flux between follicle cells, which in turn promotes endocytosis and activation of the JAK/STAT pathway (Sahu et al. 2017). A recent study demonstrates that Inx2 and Inx3 regulate microtubules in border cells, which is critical for border cells to integrate into the epithelium upon arrival at the oocyte (Miao et al. 2020). Interestingly, functions of channels or gap junctions are dispensable for this neolamination process of border cells, suggesting various roles and molecular mechanisms of gap junction proteins.

To identify roles of Inxs, we tested four Inx genes expressed in follicle cells, including ogre (Inx1), Inx2, Inx3, and Inx7 (Stebbings et al. 2002). Only Inx2 was required for regulating StC morphogenesis. Blocking of gap junction functions did not affect StC morphogenesis, suggesting that Inx2 may promote StC flattening in a gap junction-independent manner. The level of βPS encoded by mys was significantly higher in Inx2 loss-of-function StCs than that in control cells. Importantly, simultaneous knockdown of Inx2 and mys partially rescued the morphogenetic defects of StCs caused by Inx2 knockdown. Reduction of βPS induced early StC flattening. These results suggest that βPS acts downstream of Inx2 in modulating StC flattening. Interestingly, the level of microtubules was increased in Inx2 mutant cells. While microtubules are critical for integrin trafficking, this may provide a mechanism for regulation of integrins by Inx2 (Seetharaman and Etienne-Manneville 2019). Taken together, we demonstrate a novel genetic interaction between Inx2 and mys in modulating cell morphogenesis, possibly through a gap junction-independent mechanism.

Materials and methods

Fly strains

The following Drosophila strains were used for RNAi knockdown experiments:

\[
\begin{align*}
\text{Inx1 (ogre): y}^1 \text{v}1; P(\text{TRIP/JF2595}) \text{attP2 (BLM 27283)}; \text{Inx2: y}^1 \text{v}1; P(\text{TRIP/JF204246}) \text{attP2 (BLM 29306)}; \\
\text{Inx3: w1118; P(GD14965)u39094, y}^1 \text{v}1; \text{Inx7: P(TRIP/JF2066)attP2 (BLM26297), w: } u1118; P(GD15002)u29619, \text{ mys: } P(KK100518)VIE-260B(v103704)UAS-GFP. GAL4 lines: P(GauB)G306.
\end{align*}
\]

The following Drosophila strains were used for generation of mitotic clones:

\[
\begin{align*}
P(\text{neoFRT})19A, \text{ ey} = \text{flp} \\
\text{w}^{67/23}P(\text{lacW})\text{Inx2}^{G0009}P(\text{neoFRT})19A/\text{FM7c, P(ey} = \text{FLP.N)5} \\
\text{w}^{67/23}P(\text{lacW})\text{Inx2}^{G0152}P(\text{neoFRT})19A/\text{FM7c, P(ey} = \text{FLP.N)5} \\
\text{y}^{1}w^{1} \text{Inx2}^{P(\text{neoFRT})19A/\text{FM7c, P(GAL4} - \text{Kr.C)DC1, P(UAS} - \text{GFP.S657)DC5, sm}^+ \\
\text{y}^{1}w^{1} \text{Inx2}^{P(\text{neoFRT})19A/\text{FM7c, P(GAL4} - \text{Kr.C)DC1, P(UAS} - \text{GFP.S657)DC5, sm}^+ \\
\text{P(\text{hsFLP})1, P(tubP} - \text{GAL80)LL1 w^{1}P(\text{neoFRT})19A; \text{Pinf1/CyO}
\end{align*}
\]

Generation of mosaic analysis with a repressible cell marker clones

The FLP/FRP site-specific recombination system was applied to generate homozygous GFP-positive mutant clones with a heat-shock promoter (Wu and Luo 2006). Newly eclosed flies were collected for heatshock at 37°C for four times in constitutively 2 days. On the first day, flies were heatshocked twice for 30 minutes with a 3-hours interval between heatshocks; on the second day, flies were heatshocked once for 30 minutes and once for 60 minutes with a 3-hours interval between heatshocks. All flies were cultured at 25°C for 6 days before dissection.

RNAi experiments

Flies were raised at 18°C before eclosion. Newly eclosed adult flies were collected and grown at 29°C for 6 days before dissection.
Egg chambers culturing and application of Gap junction blockers

Drosophila ovaries were dissected in Schneider’s medium containing 15% fetal bovine serum, 0.6 x penicillin/streptomycin, and 0.10 mg/ml insulin (Prasad et al. 2007). Medium was maintained at 25°C and replaced every 1 hour and a half. Carbenoxolone was dissolved in water and used at 0.2 mM (Speder and Brand 2014). Ovaries were fixed as described below.

Immunofluorescence staining and microscopy

Ovaries were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS for 15 minutes. After fixation, ovaries were washed with PBT (1XPBS, 0.5% Triton X-100) for 3 times. Next, ovaries were incubated in the blocking solution PBTB (1XPBS, 0.5% Triton X-100, 5% goat serum, 2.5 mg/ml BSA, and 0.05% Sodium azide) at room temperature followed by incubation in PBTB with the primary antibodies for overnight at 4°C. Ovaries were then washed with PBT and incubated with secondary antibodies for overnight at 4°C. The following antibodies were used: mouse anti-Armadillo (1:200 dilution, N2 7A1 Armadillo, DSHB), mouse anti-Discs large (1:200, 4F3 anti-discs large, DSHB), mouse anti-Integrin βPS (1:200, CF6G11, DSHB), mouse anti-Cut (1:200, 2B10, DSHB), mouse anti-Hindsight (1:200, 1G9, DSHB), mouse anti-Fasciclin II (1:200, 1D4 anti-Fasciclin II), rat anti-DE-cadherin, extracellular domain (1:200, DCAD2, DSHB), rabbit anti-GFP (1:1000, Invitrogen), guinea pig anti-Inx2 (1:1000, provided by Dr. Guy Tanentzapf), anti-α Tubulin (1:1000, DM1A, Sigma-Aldrich), anti-β Tubulin (1:200, AA12L.1, DSHB), Dylight-488 goat anti-rabbit IgG (H + L), Dylight-549 goat anti-mouse IgG (H + L), Dylight-549 goat anti-guinea pig IgG (H + L) (Jackson Immunoresearch Laboratories), and Alexa Fluor 633 goat anti-mouse IgG (H + L) (Invitrogen). Ovaries were further stained with DAPI in PBT (1μg/ml, Sigma) prior to mounting with mounting solution [85% glycerol, 1XPBS, 3% propyl gallate (Sigma), and Prolong Gold Antifade reagents (Invitrogen, Carlsbad, CA, USA)]. All images were taken by using Zeiss LSM700 (Carl Zeiss AG, Germany). The images were further arranged and optimized by using Adobe Photoshop CS3 (San Jose, CA, USA).

Images analysis and quantification

Single slice of confocal images were used for analysis of the fluorescence intensities. The fluorescence intensities were measured by using ImageJ (NIH, USA). The value of “Mean Intensity” in ImageJ was used. One to three Inx2 mutant cells and adjacent control cells of the same picture were selected for measurement. At least seven images with marked clones were measured for each genotype. The relative fluorescence intensities in the apical region of DE-cad, Arm, or βPS, and the lateral region of α and β Tubulin, were calculated. Stages 9 and 10 egg chambers were selected for measurement of the distance between StC nuclei in a single confocal plane. The distance between adjacent StC nuclei was measured and calculated by using the line tool in Zen (blue edition, Zeiss, Germany). For quantification of the fluorescent intensity of stat::GFP, egg chambers at stage 8 were selected. Those border cells adjacent to the anterior polar cells with stronger GFP intensities in a confocal plane were selected for measurement. The mean intensity of the cytoplasmic region of the individual cell was measured. The mean intensity of the cytoplasmic region of polar cells was used as background for subtraction.

Proximity ligation assay

Ovaries were dissected in PBS, fixed in 4% PFA in PBS and followed by immunofluorescence staining protocol for incubation of primary antibodies. After washing, the manufacturer’s protocol (Sigma-Aldrich/Thermo Fisher USA) modified for whole-mount tissue staining was followed (Wang et al. 2015). Goat anti-guinea pig IgG (Invitrogen, USA) was conjugated with Plus oligonucleotide following the manufacturer’s protocol (Duolink in situ Probesmaker Plus, Sigma-Aldrich/Thermo Fisher USA). Single slice of confocal image was used. For quantification of proximity ligation assay (PLA) data, punctate staining signals were counted.

Results

Inx2 is required for StC morphogenesis

mRNAs of four gap junction protein genes, ogre (Inx1), Inx2, Inx3, and Inx7, are detected in follicle cells during oogenesis (Stebbings et al. 2002). To investigate roles of gap junction proteins in follicle cells, we used c306-GAL4 to drive expression of shRNA in follicle cell precursors, anterior/posterior follicle cells, polar cells, StCs, and border cells (Supplementary Figure S1). UAS-GFP driven by c306-GAL4 was used as a control. Immunofluorescence of DE-cad was used for labeling the apical-lateral membrane of follicle cells and border cell clusters (Geisbrecht and Montell 2002; Yeh et al. 2015). Knockdown of Inx2 driven by c306-GAL4 did not affect follicle cell differentiation during early oogenesis (data not shown). During StC flattening at stage 9, the distance between StC nuclei was increased dramatically as shown in the UAS-GFP control and Inx1, Inx3, and Inx7 knockdown groups (Figure 1, A, B, D, and E). When Inx2 was knocked down, StCs nuclei were distributed close to one another (Figure 1C, C’, Supplementary Table S1), suggesting attenuation of StC morphogenesis. Inx2 has been reported to regulate border cell fate by controlling the JAK/STAT signaling (Sahu et al. 2017). Consistently, knockdown of Inx2 driven by c306-GAL4 resulted in defective border cell migration (Figure 1C and data not shown). To confirm the phenotype of Inx2 loss-of-function, we used mosaic analysis with a repressible cell marker (MARC3) to generate Inx2 mutant clones (Wu and Luo 2006). As a control, FRT15A GFP-positive StCs underwent cell flattening and their cell nuclei were sparsely distributed at stage 9 (Figure 1, F and F’). The cell nuclei of GFP-positive Inx2 mutant StCs remained tightly distributed (Figure 1, G and G’). Inx2 mutant border cells formed a cluster successfully but failed to detach from StCs (Figure 1G), demonstrating that those tightly distributed Inx2 mutant cells surrounding the nurse cells are StCs that failed to flatten, but not border cells. Taken together, these data show that Inx2 is required for StC morphogenesis.

Inx2 is distributed at both apical and lateral membrane of follicle cells

We next examined the distribution of Inx2 in the ovary by using immunofluorescence. Inx2 was not detected in GFP-positive Inx2 mutant cells (Supplementary Figure S2A), demonstrating the specificity of anti-Inx2 antibody (anti-Inx2) (Smendziuk et al. 2015). Immunofluorescence of Disc large-1 (Dlg) was used for labeling the lateral membrane of follicle cells (Yeh et al. 2015). Inx2 was detected on the membrane between germline cells (Supplementary Figure S2, B, C, and C’). In follicle cells, Inx2 was mainly detected on the apical membrane during early oogenesis (Supplementary Figure S2, B, C, and C’). At stage 9, puncta of anti-Inx2 staining were observed at both apical and lateral membrane of follicle cells (Figure 1, H, H’, and H”), demonstrating that Inx2 is detected in StCs.
Figure 1: Inx2 is required for StC morphogenesis. Egg chambers at stage 9 were selected and oriented as anterior to the left. Ovaries were stained with anti-DE-cad (A–E), anti-GFP (A, F, and G), anti-Dlg (H), anti-Inx2 (H), and DAPI for DNA in cell nuclei. High magnification views are shown in (C', F', G', H', and H''). (A–E) Newly eclosed flies were grown at 29°C for 6 days before dissection. UAS-RNAi targeting Inxs were driven by c306-GAL4. UAS-GFP driven by c306-GAL4 was used as a control. StC nuclei are indicated by white arrowheads. (A, B, D, and E) In control, Inx1, Inx3, and Inx7 knockdown groups, StCs became squamous and their nuclei were separated from one another. (C) In Inx2 knockdown group, StCs were not flattened and their nuclei were close to one another. The border cell cluster failed to form (the yellow arrow). (F, F', G, and G') GFP-positive FRT19A or Inx2G0059 mutant clones (yellow dashed lines) were generated by using MARCM and examined 6 days after clone induction. StC nuclei are indicated by white arrowheads. (F) In FRT19A control, nuclei of StCs were sparsely separated from one another. (G) Nuclei of Inx2 mutant StCs were close to one another. The border cell cluster failed to detach from StCs (the yellow arrow). (H) White brackets indicate the regions shown in (H') and (H''). Inx2 was detected on both apical membrane (arrows) and lateral membrane (arrowheads) of StCs during morphogenesis. Length of the scale bar is 20 μm.
Blocking of gap junctions with a gap junction blocker carbenoxolone is not sufficient to attenuate StC morphogenesis

Since Inx2 is detected on both the apical and lateral membrane of follicle cells (Figure 1H), Inx2 may form gap junctions for intercellular communication between follicle and germline cells and/or among follicle cells. Thus, we examined whether the gap junction activity was required for StC morphogenesis by using a gap junction/channel blocker carbenoxolone. Carbenoxolone has been applied to block gap junctions in the nervous system and ovary of Drosophila successfully (Sperer and Brand 2014; Sahu et al. 2017; Miao et al. 2020). It takes approximately 6 hours for StC morphogenesis from stages 9 and 10 (Horne-Badovinac and Bilder 2005), so we cultured ovaries from wild-type flies for 3 or 6 hours in Schneider medium containing either carbenoxolone or vehicle control and examined whether StC morphogenesis was affected. Unexpectedly, incubation in carbenoxolone did not change the distribution of StC cell nuclei compared with that of the vehicle control at stages 9 and 10 (3 hours in Figure 2, A and B and 6 hours in Supplementary Figure S3, A and B). To test the effectiveness of carbenoxolone treatment, we examined the fluorescent intensity of stat::GFP, a reporter for the JAK/STAT pathway. The immunofluorescent intensity of stat::GFP has been shown to be regulated by gap junction formation of Inx2 (Sahu et al. 2017). After incubation in 200 μM carbenoxolone for 4 hours, the stat::GFP immunofluorescent intensity was significantly reduced in border cells (Supplementary Figure S3, C–F), demonstrating the effectiveness of carbenoxolone treatment in suppressing gap junction functions in our ex vivo culture system. Thus, in addition to gap junction formation, Inx2 might modulate StC morphogenesis through a gap junction-independent mechanism.

Defective StC morphogenesis in Inx2 mutants is not caused by delay of follicle cell differentiation

Defective StC morphogenesis may be caused by delay of follicle cell maturation, which subsequently leads to postponement of StC morphogenesis. Prior to StC morphogenesis during stage 6 to 7, the Notch pathway regulates the mitosis to endocycle (M/E) transition of follicle cells through up-regulation of a zinc-finger transcription factor Hindsight (Hnt) and down-regulation of a homeodomain protein Cut (Deng et al. 2001; Lopez-Schier and St Johnston 2001; Sun and Deng 2005, 2007). To exclude the possibility that maturation of Inx2 mutant follicle cells is delayed, we examined the M/E transition of Inx2 mutant cells. In FRT19A control at stage 9, Hnt was detected in StCs, border cells and posterior follicle cells; Cut was only detected in polar cells (Figure 2, C, C’, E, and E’). Despite failure of morphogenesis, Inx2 mutant StCs was positive for Hnt and negative for Cut as that of the adjacent control StCs (Figure 2, D, D’, F, and F’), demonstrating that Inx2 mutant cells undergo M/E transition normally. Therefore, it is unlikely that the defect of morphogenesis in Inx2 mutant StCs is caused by delay of follicle cell maturation.

The TGF-β pathway may not act downstream of Inx2 in regulating StC morphogenesis

A previous study has demonstrated that the TGF-β pathway promotes StC morphogenesis through remodeling of adherens junctions and cytoskeletons (Brigaud et al. 2015). To test whether Inx2 interacts with the TGF-β pathway during StC morphogenesis, we examined the activity of the TGF-β pathway by a Dad-lacZ (Dad, Daughter against dpp) enhancer trap line. The TGF-β pathway is activated in germline stem cells and follicle cells during StC morphogenesis and centripetal migrating cells at stage 10B (Kai and Spradling 2003). The expression of Dad-lacZ was barely detected in Inx2 mutant FSCs and follicle cells as well as adjacent control cells in the gerarium at stage 2 (Supplementary Figure S4A). Interestingly, Dad-lacZ was up-regulated from stages 4 to 7 in Inx2 mutant follicle cells comparing with the GFP-negative neighboring control cells (Supplementary Figure S4, B and C). Strong expression of Dad-lacZ was detected in Inx2 mutant StCs and main body follicle cells at stage 10 (Supplementary Figure S4D). Since activation of the TGF-β pathway promotes StC morphogenesis (Brigaud et al. 2015), it is unlikely that Inx2 promotes StC morphogenesis through inhibiting the TGF-β pathway.

Inx2 regulates βPS in StCs during morphogenesis

We next examined whether Inx2 modulates StC morphogenesis through regulating actin cytoskeletal rearrangement, cellular junctions or cell adhesion. FRT19A and Inx2 mutant MARCM clones were generated and stained for actin cytoskeleton or components of junctional and adhesion complexes. Single slice of confocal images were used for image analysis. Neither the level nor the distribution of filamentous actin was changed in GFP-positive Inx2 mutant cells in comparison with FRT19A control clones or neighboring GFP-negative control cells (Figure 3, A and B), suggesting that Inx2 does not regulate StC morphogenesis through rearrangement of the actin cytoskeleton. It has been reported that Inx2 is required for epithelial morphogenesis in the Drosophila embryo through interaction with components of adherens junction complex, such as DE-cad and Arm (Bauer et al. 2004). In addition, the Ser/Thr kinase Tao promotes endocytosis of a cell adhesion molecule Fas2 on the lateral domain to relieve intercellular connection and facilitate StC morphogenesis (Gomez et al. 2012). Thus, we examined the level and distribution of DE-cad, Arm, and Fas2. In FRT19A control clones at stage 9, DE-cad or Arm was detected on the apical-lateral domain of follicle cells and StCs (Figure 3, C, C’, E, and E’). The immunofluorescent intensity of DE-cad in GFP-positive Inx2 mutant cells were slightly increased comparing with the neighboring GFP-negative control cells (Figure 3, D and D’, Supplementary Figure S5). The level and distribution of Arm in Inx2 mutant cells were not significantly different from that of the neighboring GFP-negative control cells (Figure 3, F and F’, Supplementary Figure S5). Fas2 was enriched in polar cells and barely detected in FRT19A control StCs at stage 9 (Figure 3, G and G’). The level and distribution of Fas2 in Inx2 mutant cells were similar to that of the neighboring GFP-negative control cells (Figure 3, H and H’). Taken together, Inx2 may not regulate StC flattening through modulating actin cytoskeleton, Arm, or Fas2-mediated lateral adhesion. Inx2 may regulate StC morphogenesis through modulating DE-cad. Since the fold-change of DE-cad immunofluorescent intensity in Inx2 mutant cells is only 1.33 fold (Supplementary Figure S5), this regulation might not be the primary mechanism for the StC morphogenetic defect in Inx2 mutant cells.

During oogenesis, the composition of integrins surrounding follicle cells changes dynamically (Dinkins et al. 2008; Delon and Brown 2009). Integrins are required for the morphogenesis from cuboidal to columnar shape of posterior follicle cells (Ng et al. 2016). We examined the distribution of the β subunit βPS in follicle cells during oogenesis. Single slice of confocal images were used for image analysis. βPS was detected on the apical domain of follicle cells prior to stage 6 (Supplementary Figure S6, A and B). From stages 7 to 8, βPS was detected on the apical-lateral domain of follicle cells (Supplementary Figure S6C). At stage 10, βPS
is down-regulated in StCs and relocated to the basal domain in the posterior columnar follicle cells (Supplementary Figure S6D). We, therefore, speculated that βPS might play a role downstream of Inx2 in modulating StC morphogenesis. Prior to stage 9, the distribution and level of βPS in Inx2 mutant follicle cells were the same as that of the neighboring GFP-negative control cells (Figure 4, A, B–B′). At stage 9, the level of βPS was significantly higher in Inx2 mutant StCs than that of the FRT19A control StCs or the neighboring GFP-negative control cells (Figure 4, C, C′, D, and D′). Supplementary Figure S5). βPS was accumulated on the apical and lateral membrane of Inx2 mutant StCs (Figure 4D′). Furthermore, increase of βPS was observed specifically in Inx2 mutant StCs but not in Inx2 mutant posterior follicle cells (Supplementary Figure 4D′). By using confocal microscopy to examine the surface plane the egg chamber, the level of βPS in Inx2 mutant StCs was significantly higher than that of the neighboring GFP-negative control cells (Figure 4, E and E′). Our result demonstrates that Inx2 deficiency leads to an increase of βPS specifically in StCs during morphogenesis.

**Genetic interaction between βPS and Inx2 in regulating StC morphogenesis**

If excessive βPS in Inx2 loss-of-function follicle cells attenuates StC morphogenesis, reduction of βPS may rescue the morphogenetic defect. We used c306-GAL4 to drive shRNA expression. UAS-GFP was used as a control. In the control group at stage 9, StCs flattened and their cell nuclei were sparsely distributed (Figure 5, A and A′). Consistent with Figure 4D, knockdown of Inx2 led to defective StC morphogenesis and an increase of βPS (Figure 5, B and B′). When mys was knocked down, StCs flattened normally at stage 9 (Figure 5, C and C′). The level of βPS in follicle cells at both anterior and posterior parts of the egg chamber was significantly reduced, demonstrating the efficiency of mys knockdown (Figure 5, C and C′). When Inx2 and mys were knocked down simultaneously, flattened StCs were observed in many egg chambers (Figure 5, D and D′). Quantification analysis showed that knockdown of mys and Inx2 significantly reduced the percentage of egg chambers containing aberrant StCs in comparison with knockdown of Inx2 alone (Figure 5E), suggesting a partial rescue. To further quantify the phenotype of StC morphogenesis, we plotted the distance between adjacent StC nuclei as histograms (Figure 5F). The median for c306>GFP, c306>Inx2RNAi, c306>mysRNAi, and c306>mysRNAi; Inx2RNAi is 22.6, 6.5, 14.3, and 8.7 μm, respectively. In comparison with the distribution of c306>GFP control, the distribution of c306>Inx2RNAi dramatically shifted to the left (Figure 5F). The distribution and peaks of c306>Inx2RNAi; mysRNAi shifted to the right in comparison with the distribution of c306>GFP (Figure 5F). To rule out the possibility that the partial rescue in Figure 5D has resulted from dilution of c306-GAL4, we used c306-GAL4 to drive expression of both GFP and Inx2 shRNA. Knockdown of mys and Inx2 significantly reduced the percentage of egg chambers containing aberrant StCs in comparison with knockdown of Inx2 and expression of GFP simultaneously (Figure 5E and Supplementary Figure S7). This result suggests that accumulation of βPS in Inx2 loss-of-function cells may contribute to the attenuation of StC morphogenesis.
Down-regulation of βPS induces early StCs morphogenesis

If abnormal accumulation of βPS attenuates StC morphogenesis, reduction of βPS may induce StC flattening. To test this hypothesis, we used c306-GAL4 to drive shRNA expression. Two mys RNAi lines with different shRNA targeting sequences were used. Egg chambers with oocyte occupying 21–30% of the egg chamber length were selected when most StCs in the control group remain cuboidal shape and the cell nuclei were tightly distributed (Figure 6, A and A’). More egg chambers in mys knockdown groups contained flattened StCs at this stage comparing with that of the control (Figure 6, B, B’, C, C’, and C’). Quantitative analysis demonstrated that StC morphogenesis was observed in 25.7% of the egg chambers in c306>GFP control; 75 and 64.3% of the egg chambers in c306>mysRNAi v29619 and v103704, respectively. To further demonstrate the phenotype of StC morphogenesis, we plotted the distance between adjacent follicle cell nuclei as histograms (Figure 6E). Six of the most anterior cell nuclei were selected for each egg chamber. The mean ± standard deviation (SD) for c306>GFP, c306>mysRNAi v29619 and v103704 is 6.9 ± 2.86, 8.93 ± 6.10, and 8.65 ± 6.33 µm, respectively. In addition to the increase of means in both mys knockdown groups, the SD for both mys knockdown groups was higher than that of c306>GFP control group, suggesting a more spread distribution of the distance between adjacent follicle cell nuclei when mys was knocked down (Figure 6E). Because βPS was down-regulated at stage 9 during StC morphogenesis (Figure 4C, Supplementary Figure S6), our data suggest that down-regulation of βPS may contribute to StC flattening.

Inx2 regulates microtubules during StC morphogenesis

Microtubules are critical for integrin trafficking (Seetharaman and Etienne-Manneville 2019). A recent study demonstrates that the level of microtubules was significantly decreased in Inx2 mutant border cells (Miao et al. 2020), so we examined whether microtubules were affected in Inx2 mutant StCs. In FRT19A control clones at stage 9, both α and β Tubulins were detected on the
Figure 4 Inx2 modulates the level of and distribution of βPS specifically in StCs. Egg chambers at different stages were selected and oriented as anterior to the left. GFP-positive FRT19A and Inx2G0059 mutant clones (yellow dashed lines) were generated by using MARCM and examined 6 days after clone induction. Ovaries were stained with anti-GFP, anti-βPS and DAPI. High magnification views are shown in the right panels (B’, B”, C’, D’, and D”). StC nuclei are indicated by white arrowheads. (A, B) The distribution and level of βPS in Inx2 mutant follicle cells at stages 2 to 3 (A), and stage 7 (B) were similar to that of GFP-negative adjacent control cells. (C, C’) In FRT19A control, βPS was barely detected in StCs. (D) Inx2 mutant StCs failed to flatten. (D’) The intensity of βPS immunofluorescent staining signal in Inx2 mutant StCs was significantly higher than that in the adjacent GFP-negative control cells. High levels of βPS were accumulated at the apical and lateral membranes of Inx2 mutant StCs. (D”) The distribution and level of βPS immunofluorescent staining signal in Inx2 mutant posterior follicle cells were similar to that of the adjacent GFP-negative adjacent control cells. (E, E’) Surface view of follicle cells at stage 9. Inx2 mutant StCs failed to flatten. The intensity of βPS immunofluorescent staining signal in Inx2 mutant StCs, but not those posterior follicle cells, was significantly higher than that in the adjacent GFP-negative control cells. Length of the scale bar is 20 μm.

Discussion

In this study, we demonstrated that Inx2 is critical for the cuboidal-to-squamous transition of StCs in the Drosophila ovary. Blocking of gap junction functions did not interfere with StC morphogenesis, suggesting that Inx2 might modulate StC flattening through a gap junction-independent mechanism. We observe increases of βPS, DE-cad, and microtubules in Inx2 mutant cells, which may contribute directly or indirectly to the morphogenetic defect of Inx2 mutant StCs. Furthermore, reduction of mys partially rescued the morphogenetic defect of Inx2 deficient StCs; reduction of mys alone induced early StC flattening. These data suggest that βPS may act downstream of Inx2 in modulating StC morphogenesis.

Among four Inxs expressed in follicle cells, only Inx2 is required for StC morphogenesis (Figure 1). A previous study has shown that both Inx2 and Inx3 are required for epithelial organization and apical-basal polarity in embryonic epidermis. Knockdown of Inx2 leads to mis-localization of Inx3, and vice versa, suggesting that Inx2 and Inx3 may form heteromeric channels (Lehmann et al. 2006). Furthermore, Inx2 regulates cell polarity through interaction with DE-cad and Arm in embryonic epidermis (Bauer et al. 2004). In our results, knockdown of Inx3 does not affect StC morphogenesis (Figure 1D), suggesting Inx2 may not function together with Inx3 in StCs. In addition, cell polarity of Inx2 loss-of-function follicle cells is not dramatically changed based on the correct distribution of DE-cad and Arm (Figure 3), suggesting that Inx2 regulates StC morphogenesis through a novel mechanism.

The TGF-β pathway has been shown to promote StC morphogenesis through remodeling of adherens junctions and cytoskeletons (Brida et al. 2015). Dux-lacZ is a well-accepted reporter reflecting the activity of the TGF-β pathway (Casnauve and Ferguson 2004). We demonstrate that Dux-lacZ was up-regulated in Inx2 mutant follicle cells (Supplementary Figure S4), suggesting that Inx2 may negatively regulate the TGF-β pathway. Since the previous report demonstrates that activation of the TGF-β pathway promotes StC morphogenesis, we ruled out the possibility that Inx2 promotes StC morphogenesis through inhibiting the TGF-β pathway. As a transcriptional target of the TGF-β pathway, Dad encodes for the Drosophila homolog of inhibitory Smad (I-Smad), which antagonizes the receptor-regulated Smads and inhibits the TGF-β pathway activity (Tsumeizumi et al. 1997; Li et al. 2017). Therefore, it remains possible that by up-regulating Dad, Inx2 inhibits the TGF-β pathway, leading to attenuation of StC morphogenesis.

Integrins play various roles during Drosophila oogenesis, including FSC maintenance (O’reilly et al. 2008), follicle cell differentiation (Gomez-Lamarca et al. 2014), anterior–posterior
polarity of the egg chamber (Diaz De La Loza et al. 2017), border cell migration (Dinkins et al. 2008; Llense and Martin-Blanco 2008), and elimination of the nurse cells (Timmons et al. 2017). Previous studies reported that knockdown of mys and reduction of Jun N-terminal kinase (JNK) signaling activity simultaneously, but not knockdown of mys alone, disrupt border cell cluster integrity and attenuates migration (Dinkins et al. 2008; Llense and Martin-Blanco 2008). In addition, a recent study demonstrates that stiffness of the basement membrane signals through integrins to modulate egg chamber elongation and StC morphogenesis (Chlasta et al. 2017). Here, we show that reduction of βPS alone induces early StC flattening. It would be interesting to further investigate how integrin signaling modulates StC morphogenesis.

We showed that blocking of gap junction functions with carbamoxolone attenuated the activity of the JAK/STAT pathway but not StC morphogenesis. These results suggest that Inx2 may modulate StC morphogenesis independently of its gap junction activity. However, specificity and efficiency are always concerns for pharmacological approaches. While we cultured ovaries ex vivo for 6 hours, it remains possible that carbamoxolone did not block gap junction functions completely in this short period of time. While the underlying molecular mechanism of Inx2 in modulating StC morphogenesis remains unclear, one possibility is that gap junction proteins serve as scaffolds for protein–protein interaction. In mammals, Cx43 is a predominant Connexin in myocardium and epithelial tissues. Cx43 interacts with structural proteins such as cytoskeletal proteins, proteins related to

Figure 5 βPS acts downstream of Inx2 in modulating StC morphogenesis. Egg chambers at stage 9 were selected and oriented as anterior to the left. Newly eclosed flies were grown at 29°C for 6 days before dissection. Ovaries were stained with anti-βPS and DAPI. High magnification views were shown in the right panels (A’–D’). StC nuclei are indicated by white arrowheads. (A) UAS-GFP driven by c306-GAL4 was used as a control. StCs flattened and nuclei were separated from one another. (B) Knockdown of Inx2 attenuated StC flattening and led to a dramatic increase of βPS in StCs. (C) Knockdown of mys reduced the level of βPS in follicle cells at anterior and posterior ends of the egg chamber. StCs flattened and nuclei were separated from one another. (D) StCs flattened when mys and Inx2 were knocked down simultaneously. The level of βPS in follicle cells at the anterior and posterior ends of the egg chamber was reduced. Length of the scale bar is 20 μm. (E) Quantitative analysis of the percentage of egg chambers with attenuated StC flattening by Fisher’s exact test of independence and post-hoc test (*P < 0.05; **P < 0.01; ***P < 0.001). Knockdown of Inx2 attenuated StC morphogenesis. Knockdown of mys partially rescued StC flattening defect in Inx2 knockdown StCs. (F) Histograms of distance between adjacent StC nuclei. c306>GFP (45 egg chambers; 402 StC nuclei distance measured), c306>lnx2RNAi (38 egg chambers; 536 StC nuclei distance measured), c306>mysRNAi (35 egg chambers; 261 StC nuclei distance measured), and c306>mysRNAi; Inx2RNAi (49 egg chambers; 427 StC nuclei distance measured). The interval of each bar is 1 μm.
trafficking or proteins in junctional complexes (Sorgen et al. 2018). Here, we observe an increase of microtubules in Inx2 mutant StCs (Figure 7). It is possible that the defect of microtubules in Inx2 deficient StCs leads to attenuation of protein trafficking and abnormal accumulation of bPS and DE-cad, therefore interfering with StC morphogenesis. However, no evidence of direct interaction between Inx2 and tubulins was found based on PLA (Supplementary Figure S8). Androcam (Acam) and CG4942 are reported to physically interact with Inx2 based on two-hybrid system (Giot et al. 2003). Acam is a testis-specific light chain for myosin VI (Frank et al. 2006). CG4942 is a membrane insertase located in the mitochondria. Neither of them is a promising candidate for StC morphogenesis. Thus, identification of proteins physically interacting with Inx2 may help us to further understand the molecular mechanism underlying StC morphogenesis.

A recent study demonstrates that Connexin 30 (Cx30) sets the orientation of astroglial motile protrusions via modulating the laminin/integrin/Cdc42 polarity pathway in a cell culture model. Over-expression of Cx30 disrupts cell polarity and reduces the level of laminin and integrins in cultured astrocytes. Importantly, over-expression of a deficient Cx30 that cannot form gap junctions still disrupts cell polarity, suggesting that Cx30-mediated regulation of astrocyte polarity does not require gap junction functions (Chezali et al. 2018). This finding is similar to our results in StCs of the Drosophila ovary. How Cx30 regulates the level of laminin and integrins in cultured astrocytes remains unclear. We and others demonstrate that Inx2 regulates microtubules in both border cells and StCs in the Drosophila ovary in gap junction-independent manners (Miao et al. 2020). Interaction between gap junction proteins and integrins may be an evolutionarily conserved mechanism in regulating various cellular behaviors.

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Data availability

All strains used are available through the Bloomington Drosophila Stock Center, KYOTO Stock Center (DGRC), and the Vienna Drosophila Resource Center (VDRC). The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplementary material is available at figshare: https://doi.org/10.25387/g3.14797689.
Conflicts of interest

The authors declare that there is no conflict of interest.

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