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ORIGINAL ARTICLE

The ecdysone receptor signalling regulates microvilli formation in follicular epithelial cells

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Abstract Epithelial morphogenesis contributes greatly to the development and homeostasis of the organs and body parts. Here, we analysed the consequences of impaired ecdysone receptor (EcR) signalling in the *Drosophila* follicular epithelium. Besides governing cell growth, the three EcR isoforms act redundantly in controlling follicle cell positioning. Flattening of the microvilli and an aberrant actin cytoskeleton arise from defective EcR signalling in follicle cells, and these defects impact on the organisation of the oocyte membrane. We found that this signalling governs a complex molecular network since its impairment affects key molecules as atypical protein kinase C and activated Moesin. Interestingly, the activity of the transcription factor Tramtrack69 isoform is required for microvilli and their actin core morphogenesis as well as for follicle cell positioning. In conclusion, our findings provide evidence of novel roles for EcR signalling and Tramtrack69 transcription factor in controlling stage-specific differentiation events that take place in the follicular epithelium.

Keywords Ecdysone signalling · EcR dominant negative · Cad99C · Oogenesis

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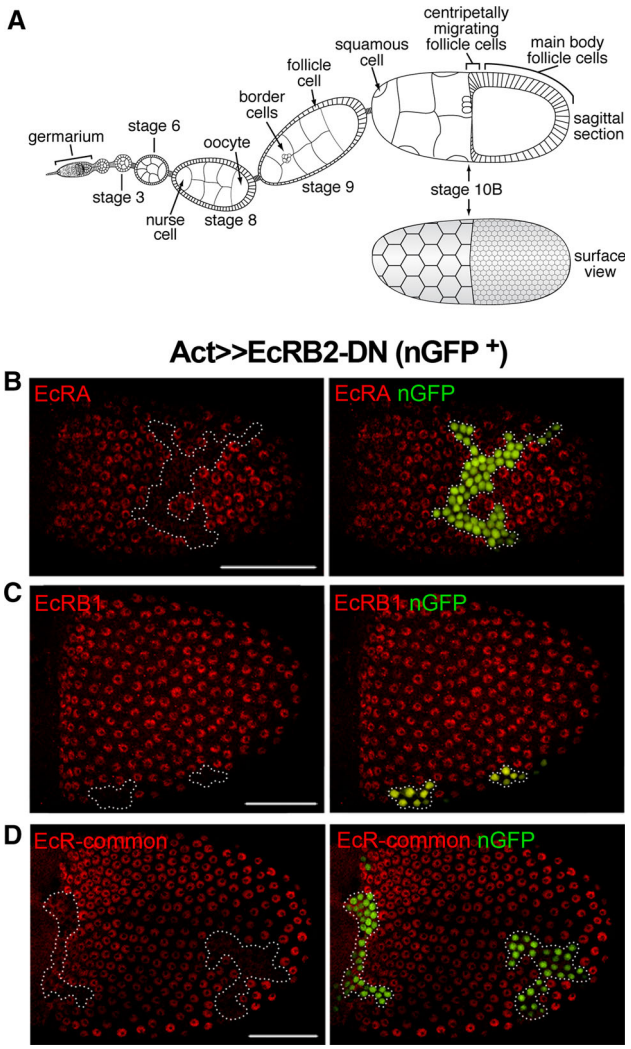
Introduction

Morphogenetic pathways are vital for the development, repair, and maintenance of adult tissues, and their disruption causes developmental problems and disease. Ecdysone hormone signalling is a key regulator of *Drosophila* development [1] and adult life [2]. This signalling is repeatedly active during oogenesis, and controls a variety of processes, such as early germline differentiation [3, 4], vitellogenesis [5] and oogenesis checkpoints [6, 7]. In fact, alterations of steroid functions trigger egg chamber degeneration. However, how this hormone elicits the cellular responses underlying oogenesis remains largely unknown.

So far, the molecular and cellular processes governing morphogenesis were studied using the follicular epithelium surrounding the developing egg chamber of *Drosophila,* as a model (Fig. 1a) [8, 9]. This epithelium arises from the progeny of follicle stem cells that undergo mitotic divisions up to stage 7 of oogenesis reaching the number of about 1,000 cells. From stage 7–9 of oogenesis, follicle cells (FCs) cease to divide and undergo three rounds of endocycling during which they replicate their genomic contents without cell division [10, 11]. Then, at stage 10B, FCs exit the endocycle and undergo amplification of specific genomic regions such as chorion genes [10]. A complex network of signalling events acts during oogenesis to define the pattern of both the follicular epithelium and the oocyte [12]. The follicular epithelium has a well-defined apico-basal cell polarity whose establishment and maintenance are required for normal tissue function. The FCs are characterised by apical, lateral and basal domains defined by the tight localisation of specific polarity complexes. The actin- and microtubule-based cytoskeletons are also highly polarised in these cells [13].



◀**Fig. 1** EcR signalling controls expression of the *EcR* gene during oogenesis. Schematic representation of a single ovariole containing a string of developing egg chambers of progressive age (**a**). The newly formed egg chamber emerges from the germarium, undergoes a continuous process of development and acquires morphological features that allow recognition of 14 different stages. In each egg chamber FCs form an epithelium that covers the nurse cells–oocyte complex. As oogenesis proceeds, complex differentiation events specify multiple subpopulation of FCs. Starting at stage 9 a group of anterior follicle cells, the border cells, delaminates from the epithelium and migrates through the nurse cells to reach the oocyte surface. As egg chamber grows, main body FCs change their shape from cuboidal to columnar and by stage 10 they cover the oocyte. The organisation of the stage 10B is illustrated in the schematic drawings of a sagittal plane through the centre of the egg chamber and of a surface view. At stage 10B few squamous FCs cover the nurse cells, the columnar main body FCs surround the oocyte and a group of anterior columnar FCs, namely the centripetally migrating FCs, starts to migrate at the interface between the oocyte and the nurse cells. The border cells after reaching the anterior region of the oocyte start to migrate dorsally. Anterior is to the *left*. The FC flp-out clones are marked by the expression of the GFP protein (*green*, *dotted area* in **b**–**d**). Confocal microscopy analysis of stage 10B egg chambers dissected from *hs-flp/Act>>Gal4; UAS-nGFP/+; UAS-EcRB2-F645A/+* females (**b**–**d**) and stained with anti-EcRA (*red* in **b**), anti-EcRB1 (*red* in **c**) and anti-EcR common (*red* in **d**), antibodies. The stage 10B egg chambers are oriented with the anterior regions to the *left*. *Scale bars* **b**–**d** 50 μm

From the beginning of oogenesis, the apical side of the FCs faces the germline cells. Throughout oogenesis, the apical domain of the FCs is characterised by the localisation of the atypical protein kinase C (aPKC), a core component of the Bazooka apical complex [14, 15]. At stage 10 of oogenesis, this domain is characterised by the presence of numerous microvilli containing the non-classical Cad99C cadherin. This transmembrane protein localises at the apical plasma membrane of the FCs where it plays a fundamental role in establishing and maintaining microvilli morphology [16, 17]. In the apical domain of the FCs, the actin cytoskeleton is regularly packed into microvilli. In contrast, in the basal domain, the actin is organised in parallel arrays. Moesin (Moe) is an apical FERM (Four-point-one, Ezrin, Radixin, Moesin) protein that cross-links apical membrane and actin and thus plays a key role in maintaining cortical actin cytoskeleton [18].

The ecdysone receptor (EcR) signalling is mediated through the EcRA, EcRB1 and EcRB2 splice variants encoded by the *EcR* gene. These isoforms differ in their amino-terminal regions and share a common carboxyl-terminal region [19]. The ecdysone active form, i.e. 20-hydroxyecdysone binds to the heterodimer composed of the EcR and the Ultraspiracle (Usp) nuclear receptors and transcriptionally regulates several response genes [20–22]. The EcRA and EcRB1 isoforms are expressed throughout oogenesis in germline and somatic follicle cells [23]. EcRB2 isoform activity during oogenesis has also been demonstrated [24].

The function of EcR isoforms has been successfully studied through targeted expression of dominant negative molecules. These species fail to activate transcription and can knock down signalling by interfering with the ecdysone-induced gene activation [24]. Analysis of the effect of dominant negative EcRB1 and EcRB2 molecules in the follicular epithelium showed that the ecdysone signalling controls stage-specific expression of eggshell genes [25, 26]. Furthermore, we have demonstrated that EcRB1 activity is required for correct polarity of follicle cells since silencing of its expression alters the distribution of apical and basolateral cell polarity markers. Knock down of EcRB1 function causes apoptotic cell death induced by caspase indicating that this isoform is required for survival of FCs at early stages of oogenesis [27]. Targeted expression of dominant negative EcRA and EcRB2 isoforms in FCs showed that block of EcR signalling in FCs delays exit from the endocycle at stage 10B. This prolonged endocycling phase is caused by the downregulation of the zinc finger Tramtrack 69 kDa isoform (Ttk69) which plays a

key role in the switch from endocycle to gene amplification phase [28]. Furthermore, Ttk69 activity is also required for the morphogenetic movements of the dorsal-appendage-forming cells, two groups of FCs that starting from stage 12 elaborate the two eggshell protrusions that facilitate gas exchange [29, 30].

This work studies the role of EcR receptor signalling during the morphogenesis of *Drosophila* follicular epithelium. Targeted block of EcR signalling was induced in FCs by reverse genetic approaches [31] to control the expression of dominant negative mutants of EcR (EcR-DNs). Our results show that the three EcR isoforms share the same functions as they control the main body FC positioning and cell growth. In addition, we found that EcR signalling and the activity of the Ttk69 isoform are required to establish the appropriate apical domain of the FCs.

Materials and methods

Fly strains

Stocks were raised on standard cornmeal/yeast/agar medium at 25 °C, and crosses were carried out at the same temperature unless otherwise stated. yw^{67c23} was used as the wild-type stock in this study. We used the following Bloomington stocks: #6468; #6469; #6470; #9449; #9450; #9451; #9452; #6872; #6869; #5137; #7; #25946; #6; #4779; #4775; #5138; #7018; #7019; #27325; #8630; #41754.

The stock *Cy2-Gal4* was kindly provided by T. Schüpbach, *Flu-HA-fs(1)ph* was from J. Casanova, *vkg-GFP/CyO* was from R. Bernardoni, *UAS-aPKC*^{CA} was from S. Campuzano.

Clonal analysis

Random expression of each transgene in FC clones was induced through the flp-out/Gal4 technique using the flp-out cassette *Act5C>CD2>Gal4* (*Act>>Gal4*) and a *heat shock-flp-recombinase* transgene (*hs-flp*). Clones were located either by nuclear targeted GFP (nGFP) or membrane-targeted GFP (mGFP) expression or by the lack of the membrane-targeted CD2 marker (CD2). Clonal overexpression of the different transgenes was obtained by crossing the appropriate fly strains at 25 °C. Freshly eclosed females of the correct genotype were collected and heat shocked three times for 1 h at 37 °C. After each heat shock these females were transferred to fresh vials with yw^{67c23} males and incubated at 25 °C. Before dissection, the flies were transferred to fresh, yeasted food daily at 25 °C for 2 days.

EcR-DN expression in flp-out clones as well as co-expression of EcR-DN and each wild-type EcR isoform have been performed in genetic backgrounds containing the same number of *UAS*-driven transgenes. This is to ensure that additional *UAS* constructs do not titrate the available Gal4. The ovaries were dissected from females of the following genotypes: *hs-flp/Act>>Gal4; +/+; UAS-EcRB2-F645A/UAS-EcRB2, hs-flp/Act>>Gal4; +/+; UAS-EcRB2-F645A/UAS-EcRB1* and *hs-flp/Act>>Gal4; +/+; UAS-EcRB2-F645A/UAS-EcRA*.

Gal4-driven expression in follicle cells

Females *tubP-Gal80*^{ts}*/+; UAS-EcRB2-F645A/tubP-Gal4* were obtained by crossing the parental strains. The crosses were performed at 18 °C and before dissection, female progeny was transferred with yw^{67c23} males to yeasted vials at 31 °C for 6 days. The females carrying the *Cy2-Gal4* driver and selected transgenes were obtained by crossing the parental strains at 18 °C. After, they were transferred with yw^{67c23} males to yeasted vials at 18 °C for 24 h and then were transferred to yeasted vials at 31 °C for 48 h before dissection.

Mosaic analysis

FC clones mutant for ttk^{1e11} loss of function allele were generated using the MARCM technique [32]. Freshly eclosed females *hs-flp, tub-Gal4, UAS-nGFP/+; +/+; FRT82B, tub-Gal80/FRT82B,* ttk^{1e11} have been collected, subjected to three 1-h heat shock at 37 °C and incubated at 29 °C for 4 days.

EdU detection

EdU staining was carried out using the Click-iT EdU Alexa Fluor 555 Imaging Kit (Invitrogen). Ovaries were dissected in Schneider's medium (Sigma) supplemented with 10 % heat-inactivated FBS (Invitrogen), 1× Penicillin–Streptomycin–Glutamine (Invitrogen) and 2.5 µg/ml Fungizone (Invitrogen). Dissected ovaries were soaked for 1 h in 20 µM EdU solution, rinsed twice in medium and fixed 20 min in 4 % formaldehyde. After several washes in 1× PBS, ovaries were permeabilised for 1 h in 1× PBS + 1 % Triton X-100 and then blocked with 3 % BSA in PBT (1× PBS + 0.1 % Triton X-100). Samples were rinsed in 3 % BSA + PBT and then incubated for 30 min at room temperature protected from light with Click-iT reaction cocktail, as indicated in the datasheet. After several washes in 3 % BSA + PBT, ovaries were mounted in Fluoromount.

Immunofluorescence microscopy

Ovaries were dissected, fixed and stained for actin, specific antigens and DNA as previously described [27]. For lectin staining, paraformaldehyde-fixed ovaries were incubated at room temperature on a rotating wheel in a blocking solution of 2 % BSA + PBT for 1 h and then incubated overnight at 4 °C in 2 % BSA + PBT containing 150 µg/ml cell membrane-specific fluorescein-*Lycopersicon esculentum* (*Tomato*) lectin (Vector Laboratories). The following primary antibodies were used: monoclonal mouse anti-CD2 1:250 (Serotec), was detected with FITC-conjugated goat anti-mouse 1:250 (Molecular Probes); anti-EcRA 1:100 (15G1a, DSHB), anti-EcRB1 1:100 (AD4.4, DSHB), anti-EcR-common 1:100 (AG10.2, DSHB), anti-Hindsight 1:30 (1G9, DSHB), anti-dMyc 1:5 (from P. Gallant) [33, 34] and anti-Myc 1:100 (Santa Cruz Biotechnology) were detected with Cy3-conjugated goat anti-mouse 1:1000 (Jackson). Monoclonal rat anti-DE-Cadherin 1:25 (DCAD2, DSHB), anti-dFoxo 1:500 (from P. Bellosta) and anti-Yolkless (from A. Ephrussi) were detected with Cy3-conjugated goat anti-rat 1:1000 (Jackson). Polyclonal rabbit anti-ξPKC 1:200 (Santa Cruz Biotechnology), anti-Cad99C 1:10,000 (from C. Dahmann), anti-pMoe 1:100 (from F. Payre) were detected with Cy3-conjugated goat anti-rabbit 1:1000 (Jackson), DyLight 649-conjugated goat anti-rabbit 1:500 (Jackson). Polyclonal guinea pig anti-Sip1 1:250 (from R. Fehon), anti-Slik 1:500 (from D. Hipfner) were detected with DyLight 649-conjugated goat anti-guinea pig 1:500 (Jackson).

Results

The block of EcR signalling downregulates EcR expression in FCs

The expression of the EcRA-DN, EcRB1-DN or EcRB2-DN mutants (Fig. S1), each containing the aminoacid substitutions F645A or the W650A [24], was separately induced using the flp-out/Gal4 technique [35]. Both mutations affect the helix 12 of the EcR ligand binding domain and thus block AF2, the transcriptional activation function that is associated with this region. The W650A mutation lies on the inner surface of the helix 12 in a position where it interferes with the ligand binding. The F645A mutation lies on the outside surface of helix 12 and abolishes transcriptional activation without affecting ecdysone ligand binding [36].

We analysed the effect of EcR-DN mutant expression in FC clones during oogenesis (Fig. 1a) by immunofluorescence confocal microscopy. The antibodies directed against the EcRA- and EcRB1-specific domains were used to detect the two isoforms, respectively [19]. The flp-out clones overexpressing the mutant constructs were also analysed using the anti-EcR-common antibody (ab) directed against the C-terminal region of the molecule (aminoacids 649–878 of the EcRB1 sequence, Fig. S1) [19]. As all mutant constructs are deleted in the carboxyl-terminal region (aminoacids 656–878 of the EcRB1 sequence, Fig. S1) [24], the common ab recognises the endogenous native EcR isoforms only. The results of the immunostaining for the EcRB2-F645A construct are shown in Fig. 1b–d. We observe a reduced amount of the EcRA (Fig. 1b) and EcRB1 (Fig. 1c) isoforms in flp-out cells expressing the mutant construct (GFP-positive cells, right hand panels). Since a decreased signal is also obtained with the anti-EcR-common ab (Fig. 1d), we conclude that the EcRB2 isoform is downregulated in these follicle cell clones. Supplementary Table 1 summarises the results obtained through confocal microscopy analysis using the six mutants.

In summary, consistent with EcR being a hormone-responsive gene [37, 38], our results show that the block of EcR activity triggers a decrease of the EcR protein possibly due to the downregulation of the *EcR* gene itself.

EcR signalling is involved in the determination of size and positioning of follicle cells

The block of EcR signalling grossly alters the migration of the border cells of the egg chamber [39] and it also affects squamous cell formation [29]. Thus, we have analysed the effect of the downregulation of EcR on the main body FCs. The experiment of Fig. 2b–c′ shows a stage 10 egg chamber stained with anti-DE-Cadherin (DE-Cad) ab and the nuclear To-Pro-3 dye. The anti-DE-Cad ab stains the adherens junctions, identifying the cellular border [15]. The GFP fluorescence that signals the expression of the EcRB2-F645A mutant construct (GFP-positive cells) shows that these cells are positioned in the anterior region of the egg chamber in a tight group (Fig. 2b, asterisks). To-Pro-3 staining (Fig. 2b) shows that the squamous cells are localised throughout the anterior region and their distribution is similar to that of squamous cells in wild-type egg chamber (Fig. 2a). The analysis of the clones expressing EcRB2-F645A shows that 88.03 % flp-out clones ($n = 117$) are incorrectly located in the anterior region of the egg chamber. In addition, the stainings with DE-Cad (left and central panels in Fig. 2c, c′) and with To-Pro-3 (right panels in Fig. 2c, c′) show that the size of the cells and the nuclei of the flp-out clones (Fig. 2c) are strongly reduced in comparison with the size of the surrounding wild-type FCs. Activation of Notch signalling at stage 7 triggers the switch from the mitotic cell cycle to the

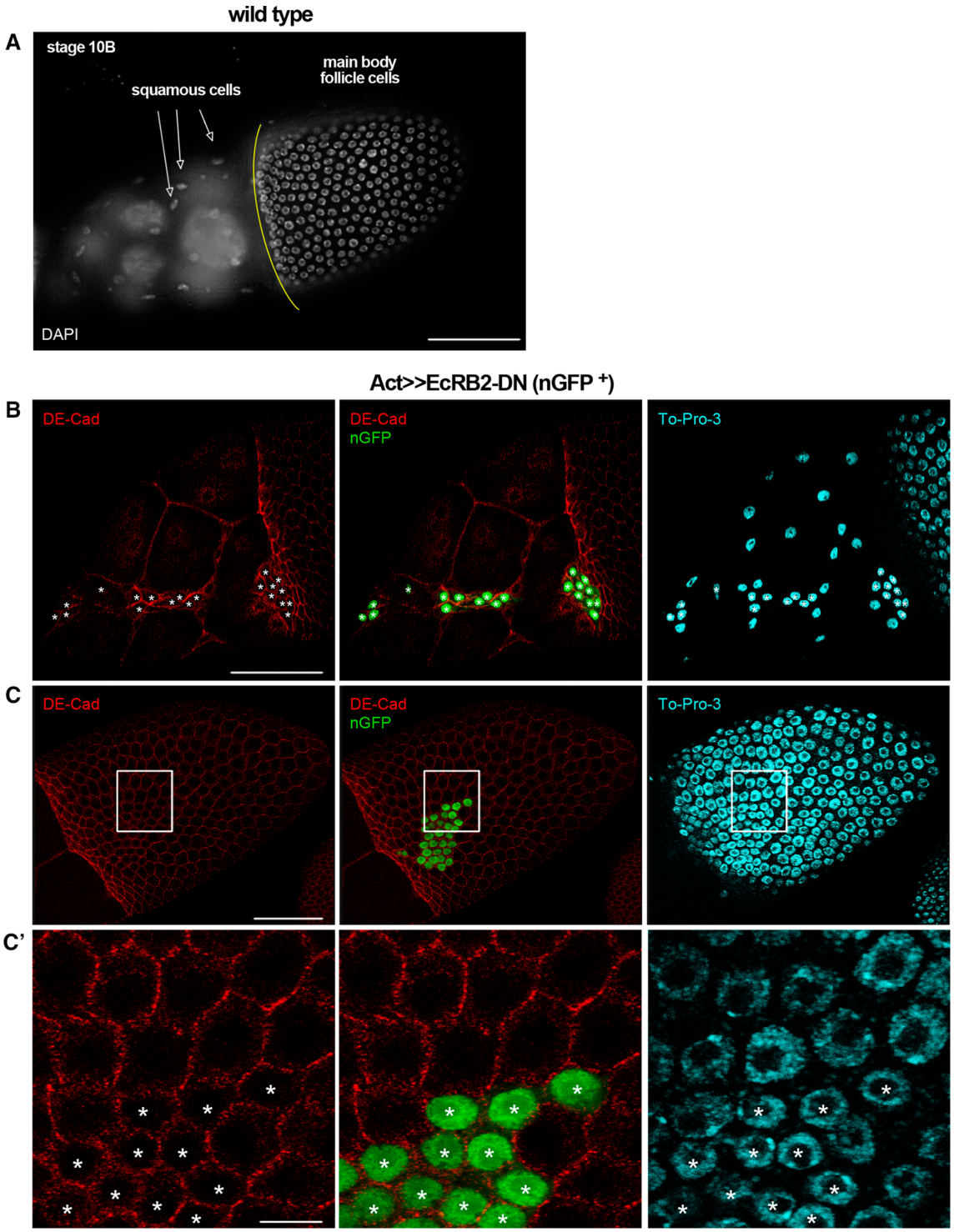


Fig. 2 Blocking EcR signalling causes mispositioning and reduced FC growth. Fluorescence microscopy analysis of a stage 10B egg chamber dissected from a wild-type female and stained with DAPI to show the positioning of the squamous FCs (*arrows*) and the main body FCs that are located in the anterior (*left* to *yellow line*) and posterior region (*right* to *yellow line*) of the egg chamber, respectively (**a**). The FC flp-out clones are marked by the expression of the GFP protein (*green*) (*asterisks*) (**b**–**c**′). Confocal microscopy analysis of stage 10B egg chambers dissected from *hs-flp/Act>>Gal4; UAS-nGFP/+; UAS-EcRB2-F645A/+* females and stained with anti-DE-Cad (*red*) ab and with To-Pro-3 nuclear dye (*cyan*). **c**′ Magnification of the *boxed* regions in **c**. The anterior region is to the *left* in all the *panels*. *Scale bars* **a**–**c** 50 μm and **c**′ 10 μm

endocycle. A second transition takes place at stage 10B when the decline of Notch signalling in the FCs triggers the transition from the endocycle to the amplification of specific genomic loci [10, 28]. The block of EcR signalling in FCs prolongs Notch activity [28]. Figure S2 shows that the reduced cell size is due to a prolonged endocycle that slows down cell growth. The experiment described in Fig. 2 was repeated with the six EcR mutants generating the same results (not shown).

The following experiments were carried out with the EcRB2-F645A mutant called for brevity EcRB2-DN. To understand if the altered cell localisation and slow cell growth observed in the previous experiments were due specifically to one of the three isoforms, we have carried out the following experiments. EcRB2-DN was co-expressed in flp-out clones with each of the wild-type EcR isoforms and the possible rescue of the phenotype was analysed. The co-expression of EcRB2-DN with EcRA ($n = 24$), EcRB2-DN with EcRB1 ($n = 28$) or EcRB2-DN with EcRB2 ($n = 16$) results in 100 % rescue of the correct position of the clones analysed at stage 10B (Fig. S3). In addition, rescue of normal FC size in flp-out clones has been detected in each of the co-expression experiments. To make sure that the overexpression of the wild-type isoforms does not alter the morphogenesis of the follicular epithelium, we have analysed flp-out clones overexpressing each isoform. No morphological damage was observed (not shown). We can conclude that all three isoforms rescue the wild-type phenotype when co-expressed with the mutant construct. This strongly suggests that the functional domain involved in rescuing the correct position of the clones is present in all three isoforms and belongs to the common carboxyl-terminal region of the molecules.

EcR signalling is involved in the organisation of the apical domain of follicle cells

We extended the clonal analysis disrupting the EcR activity through the TARGET (Temporal And Regional Gene Expression Targeting) technique [40]. The *tub-Gal4* driver was utilised to induce ubiquitous and strong expression of EcRB2-DN in the follicular epithelium. The *Cy2-Gal4* line was utilised to promote transgene expression in the follicular epithelium, from stage 7 onwards [41]. The $Gal80^{ts}$ temperature-sensitive mutant, which inhibits Gal4 activity at the permissive temperatures, was utilised to block transgene expression during development.

Figure 3a–c shows that the ubiquitous expression of the EcRB2-DN leads to the degeneration of the egg chambers throughout oogenesis. In comparison with wild-type ovarioles (Fig. 3a), DAPI staining of EcRB2-DN mutant ovarioles (97 %, $n = 215$) shows dying nurse cells and FCs with a high degree of nuclear condensation (Fig. 3b). Furthermore, mutant stage 10 egg chambers show altered distribution of the main body FCs (Fig. 3c) in comparison with distribution of the same cells in wild-type stage 10 egg chambers (Fig. 3a′). Mutant stage 10B egg chambers also show signs of degeneration (93 %, $n = 107$). The altered position of the main body FCs is particularly evident when the EcRB2-DN expression starts at stage 7 of development. We have analysed 217 stage 10 egg chambers and observed that 52.5 % of them show uneven distribution of EcRB2-DN-positive FCs which leaves the anterior surface of the oocyte uncovered (Fig. 3h, k). In contrast, the main body FCs of stage 10B control egg chamber are correctly localised and cover the oocyte (Fig. 3d–g). It should be noticed that up to 17 % of the stage 10 egg chambers expressing EcRB2-DN are degenerated ($n = 217$) (not shown).

As the movement of FCs occurs over the underlying germline cells, it seemed interesting to analyse the apical domain of FCs expressing the EcRB2-DN. We have analysed the Cad99C transmembrane protein which is essential for building and maintenance of the microvilli organisation [16, 17]. Figure 4a shows the results of staining stage 10 flp-out clones expressing the EcRB2-DN construct with specific ab. The comparison between GFP-positive clones and wild-type cells clearly shows a marked, shortening of the microvilli at a stage when the microvilli reach their maximum length in wild-type FCs. In wild-type cells, the microvilli show bundles of F-actin filaments protruding toward the oocyte, forming a regularly spaced pattern. In this region, the F-actin staining is strong in the cortex of both the FCs and the oocyte. In contrast, the F-actin filaments do not form a regular spiky pattern in the FCs expressing EcRB2-DN, but accumulate aberrantly and a reduced space separates the oocyte from the FCs. Consistent with the notion that microvilli morphogenesis in FCs starts at stage 9 of oogenesis [16, 17], the analysis of EcRB2-DN-positive FCs at this stage does not show evident alteration of the apical domain of FCs (Fig. S4). The knock down of the EcR signalling through the *Cy2-Gal4* driver results in the increased alteration of microvilli morphology and organisation. The staining with Cad99C ab shows flattened microvilli while phalloidin does not show the presence of actin bundles (Fig. 4b). In addition, the F-actin cytoskeleton of the oocyte shows aberrant architecture. In comparison with control egg chambers (Fig. 4c), the network of actin filaments repeatedly invaginates in the cytoplasm of the oocyte and forms rounded protrusions.

Since the aPKC kinase is the apical determinant [13] of FC polarity, its function may be influenced by EcR signalling. Thus, we examined the aPKC signal by confocal microscopy in flp-out FC clones expressing EcRB2-DN. Figure 5a shows that the intensity of the antibody staining of the cells expressing the mutant construct is higher than that of the wild-type cells.

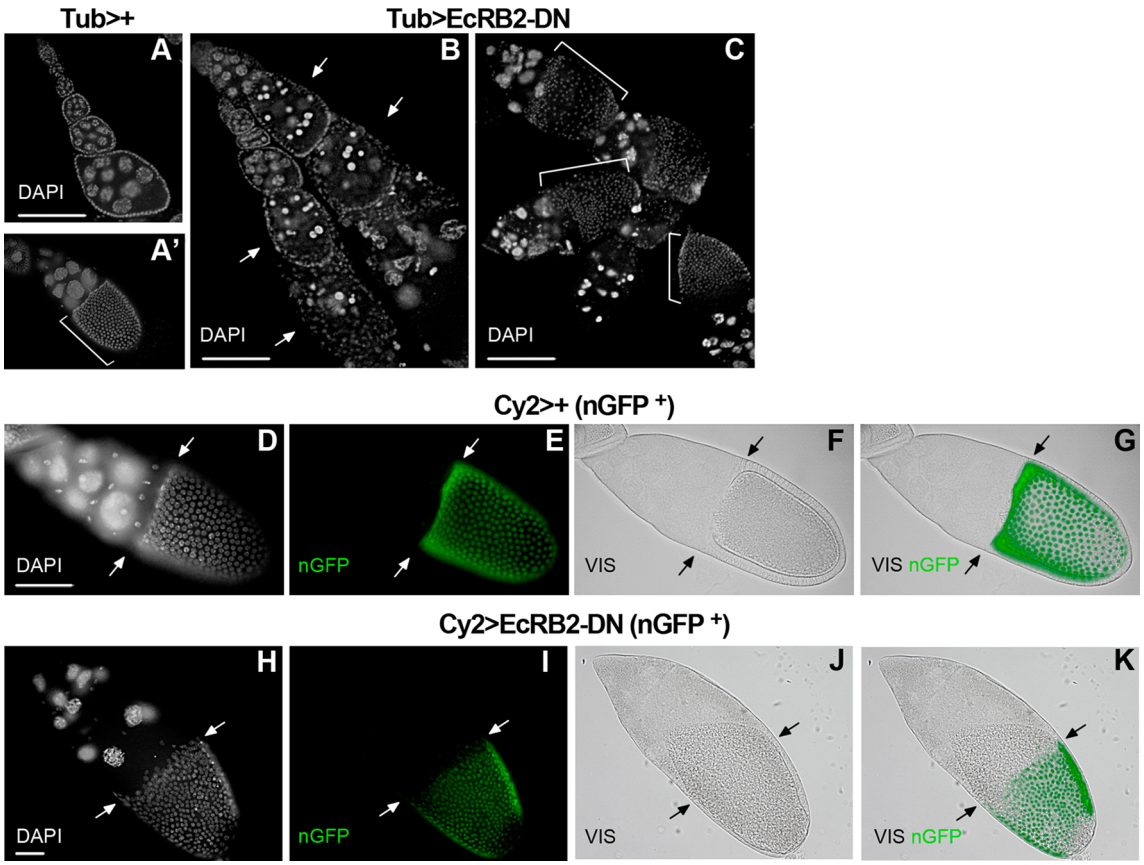


Fig. 3 Blocking EcR signalling affects egg chamber development. Fluorescence microscopy analysis of ovarioles (**a**, **b**) and stage 10 egg chambers (**a**′, **c**) dissected from *w/w; tub-Gal80^{ts}/+; tub-Gal4/+* control females (**a**, **a**′) and from *w/w; tub-Gal80^{ts}/+; tub-Gal4/UAS-EcRB2-DN* females (**b**, **c**) and stained with DAPI. The *arrows* in **b** indicate degenerating egg chambers that are absent in control ovarioles (**a**). The *brackets* in **a**′ and **c** indicate main body FCs. Fluorescence microscopy analysis of mid-oogenesis egg chambers dissected from *w/w; Cy2-Gal4/UAS-nGFP; tub-Gal80^{ts}/+* (**d**–**g**) and from *w/w; Cy2-Gal4/UAS-nGFP; tub-Gal80^{ts}/UAS-EcRB2-DN* (**h**–**k**) females and stained with DAPI. The expression domain of the *Cy2-Gal4* driver is marked by GFP (*green* in **e**, **g** and **i**, **k**). DAPI staining is in D and H (*white*). **g**, **k** Overlap of the optical images in **e**, **f** and in **i**, **j**, respectively. The *arrows* in **d**–**k** mark the limit of the main body FCs. Anterior is *up* in all *panels* with the exception of **c**. *Scale bars* **a**, **a**′, **b** 100 μm, **c** 200 μm and **d**–**k** 50 μm

It seemed interesting to find out whether the aPKC increase was influencing the microvilli structure of the EcRB2-DN-positive flp-out clones. Thus, we expressed the wild-type membrane-targeted form of aPKC, aPKC$^{CAAX\text{-}WT}$ (aPKC^{CA}) that mimics constitutive activation [42]. The staining with the anti-aPKC ab shows that the kinase construct is functional and, as expected, very abundant within the membranes where is driven by the CAAX domain (Fig. S5A). As compared with the wild-type FCs, the cells expressing the aPKC^{CA} construct show a definite decrease in size of the microvilli as shown by Cad99C staining (Fig. 5b). Contrary to the results of Fig. 4a, under these conditions, we do not observe accumulation of the F-actin filaments. We wondered whether the absence of aPKC has the opposite effect on the microvilli. Thus, we knocked down the aPKC levels through RNA interference. As compared with the wild-type FCs, the cells expressing the *UAS-aPKC-RNAi* (*aPKC-RNAi*) transgene show strongly reduced aPKC staining (Fig. S5B). In agreement with our expectations, the Cad99C staining shows that the microvilli are longer and more densely packed in the aPKC interfering clones as compared to the surrounding wild-type FCs, while the actin cytoskeleton is not grossly altered (Fig. 5c). Since these results suggest that the aPKC is involved in the control of microvilli structure, we knocked down aPKC activity by RNAi in flp-out clones expressing EcRB2-DN. This experiment would allow us to ascertain if the enhanced aPKC level in EcR mutant FCs was by itself causative of microvilli flattening. These conditions do not rescue the correct phenotype of the microvilli and of the actin cytoskeleton (Fig. 5d), suggesting that additional components act in controlling morphogenesis of microvilli.

Since the block of EcR signalling determines mislocalisation of FCs, we have analysed the clones co-expressing the *EcRB2-DN* and *aPKC-RNAi* transgenes. Under these conditions, 93.8 % flp-out clones ($n = 81$) from stage 10 egg chambers are correctly localised (Fig. S6).

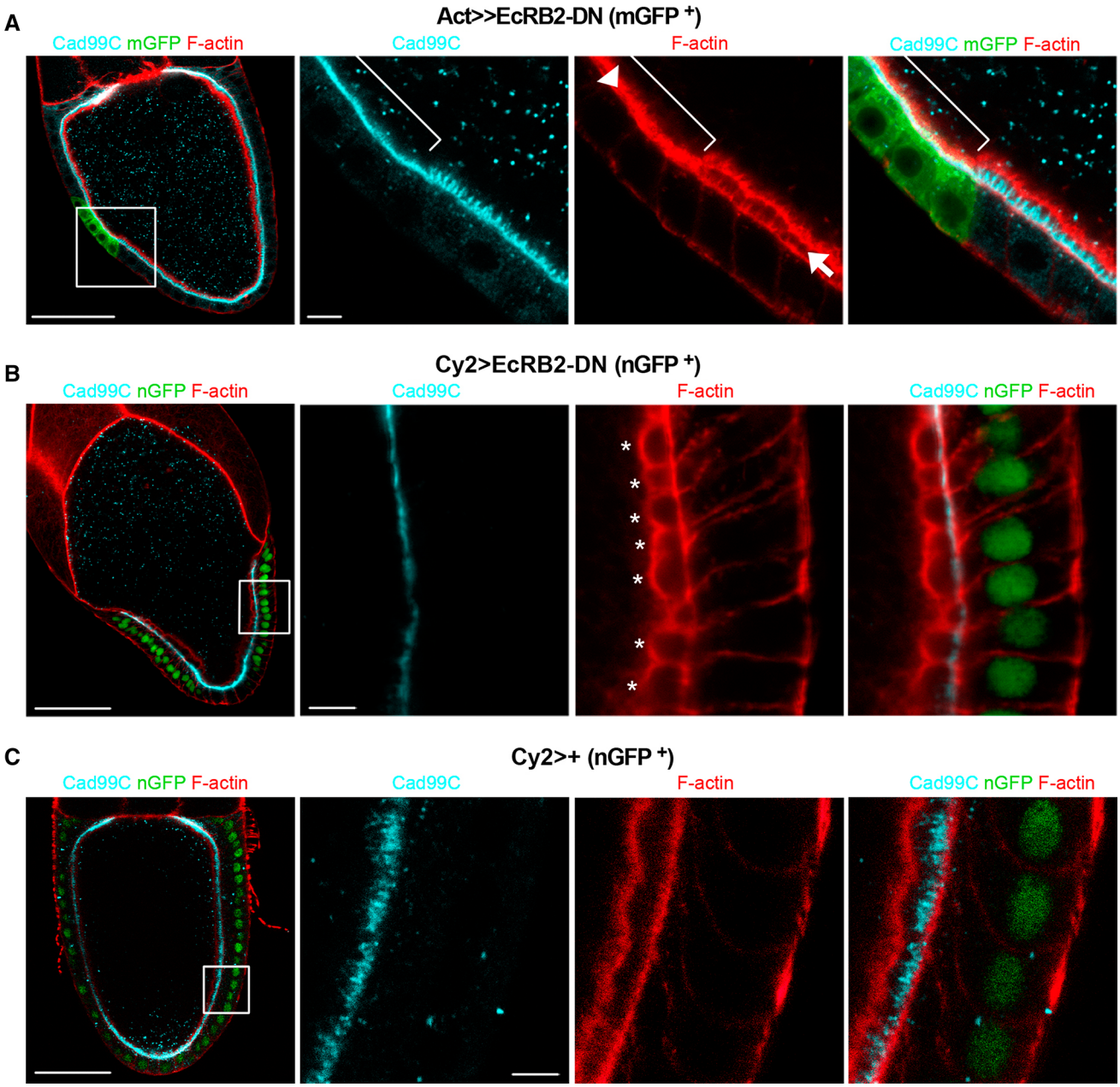


Fig. 4 Blocking EcR signalling alters microvilli and the F-actin cytoskeleton. The FC flp-out clones expressing EcRB2-DN are marked by the expression of the GFP protein (**a**) (*green*). The expression domain of the *Cy2-Gal4* driver is marked by GFP (*green* in **b**, **c**). Confocal microscopy analysis of a stage 10B egg chamber from *hs-flp/Act>>Gal4; UAS-mGFP/+; UAS-EcRB2-DN/+* females (**a**). The microvilli are stained with anti-Cad99C ab (*cyan*) and actin with phalloidin (*red*). **a** From *left* to *right* show: (1) the merging of the three signals, (2) the magnification of the microvilli, and (3) of the actin staining alone, and (4) the magnified merging of the signals. The *boxed* region in the *left panel* marks a flp-out clone with flanking wild-type cells and indicates the magnified regions on the *right*. The *brackets* indicate the flp-out clone. The *arrow* shows the space between FCs and oocyte, the *arrowhead* shows the absence of this space. Confocal microscopy analysis of egg chambers dissected from *w/w; Cy2-Gal4/UAS-nGFP; tub-Gal80^{ts}/UAS-EcRB2-DN* (**b**) and from *w/w; Cy2-Gal4/UAS-nGFP; tub-Gal80^{ts}/+* (**c**) females. The organisation and stainings of the *panels* in **b** and **c** are the same as in **a**. *Asterisks* indicate the alteration of the actin cytoskeleton. Anterior is *up* in all *panels*. *Scale bars* **a**–**c**, *left* 50 μm and **a**–**c** magnifications 5 μm

In conclusion, aPKC knock down in EcRB2-DN-positive flp-out clones suppresses mispositioning of FCs while the size of microvilli is not rescued.

The polarised architecture of the follicular epithelium also depends on the deposition of the basement membrane (BM) at the basal side of FCs [43]. Thus, we have analysed

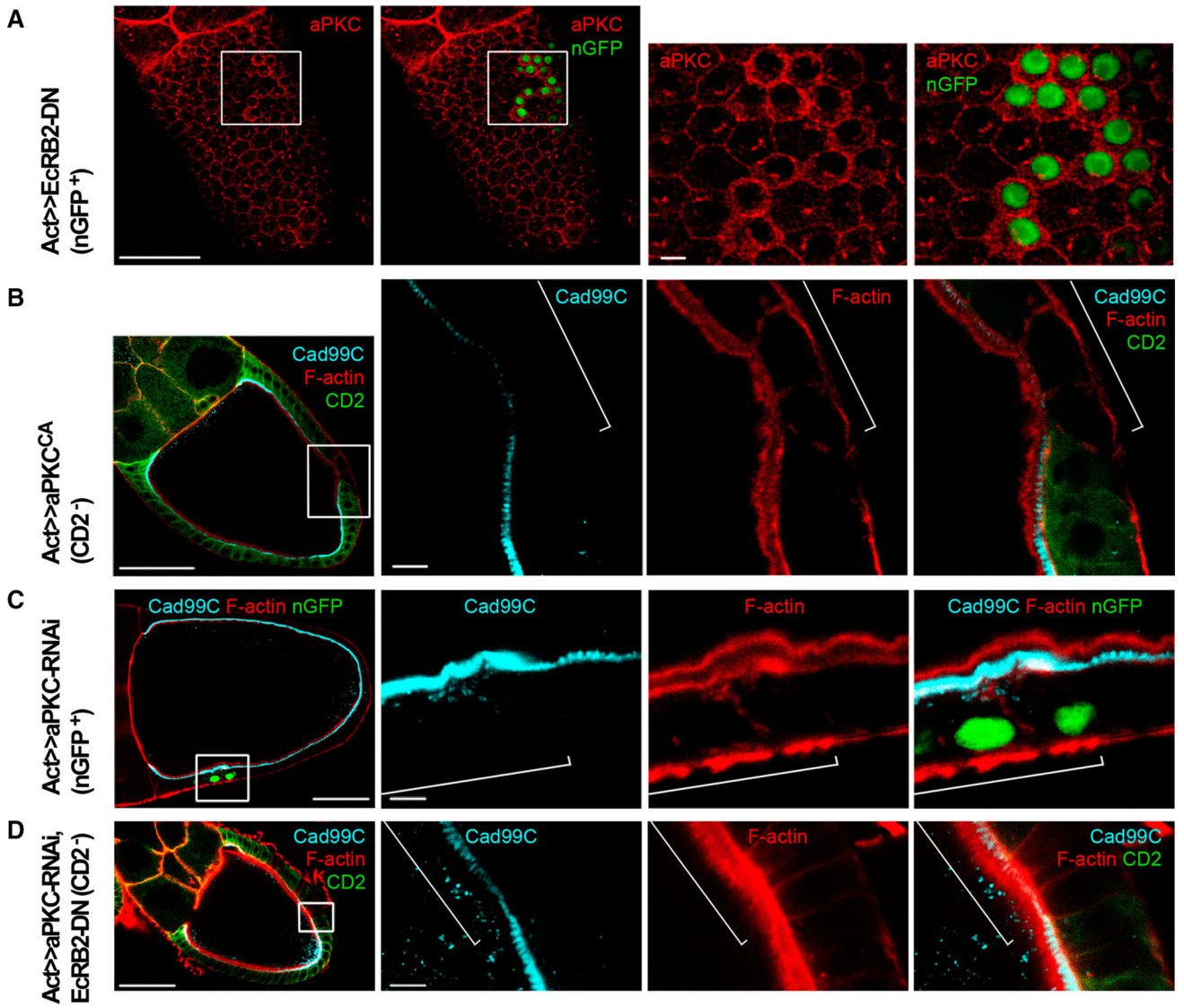
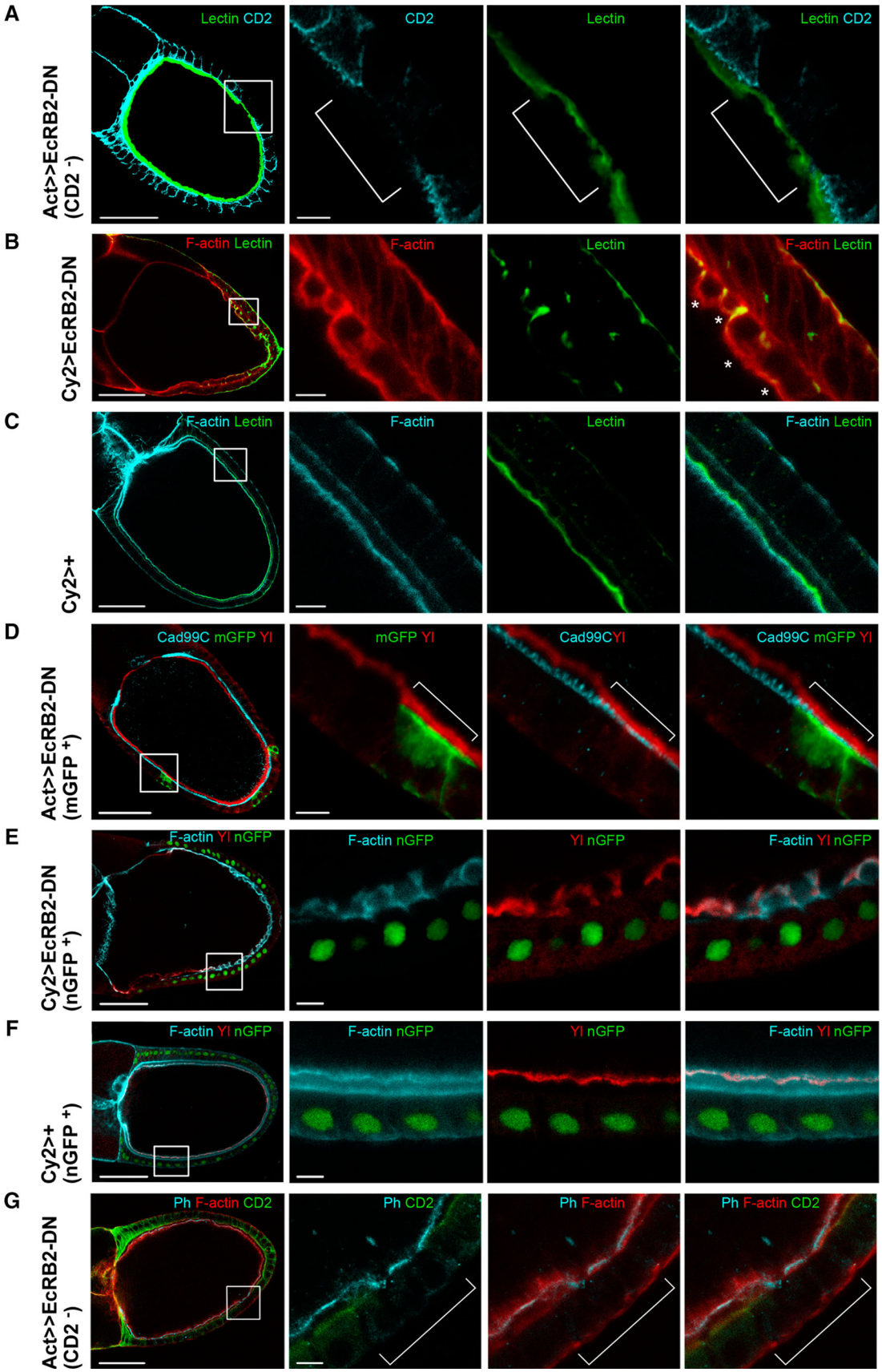


Fig. 5 The morphogenesis of microvilli requires aPKC. Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; UAS-nGFP/+; UAS-EcRB2-DN/+* stained with anti-aPKC ab (*red*) (**a**). The FC flp-out clone expressing EcRB2-DN is marked by the expression of the GFP protein (*green*). Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; UAS-aPKC^{CA}/+; +/+*, stained with anti-Cad99C ab (*cyan*), anti-CD2 ab (*green*) and phalloidin (*red*) (**b**). The FC flp-out clone expressing aPKC^{CA} is indicated by the absence of CD2 and is marked by the *brackets*. Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; UAS-nGFP/+; UAS-aPKC-RNAi/+* female stained with anti-Cad99C ab (*cyan*) and phalloidin (*red*) (**c**). The FC flp-out clone expressing aPKC-RNAi is marked by the expression of the GFP protein (*green*, *brackets*). Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; +/+; UAS-aPKC-RNAi/UAS-EcRB2-DN* female stained with anti-Cad99C ab (*cyan*), anti-CD2 ab (*green*) and phalloidin (*red*) (**d**). The FC flp-out clone expressing EcRB2-DN and aPKC-RNAi is indicated by the absence of CD2 (*brackets*). Anterior is *up* in **a**, **b**, **d** and on the *left* in **c**. All *boxed* FC flp-out clones are magnified in the *panels* on the *right*. *Scale bars* **a**–**d**, *left* 50 μm and **a**–**d** magnifications 5 μm

the distribution of the collagen IV, a secreted component of BM, in EcRB2-DN mutant FCs. We used the GFP-tagged Viking (collagen IV) trap protein (Vkg-GFP) [44]. The comparison between stage 10 egg chambers expressing the *UAS-EcRB2-DN* transgene under the control of the *Cy2-Gal4* driver and wild-type stage 10 egg chambers shows that the Vkg-GFP is normally synthesised and deposited on the basal side of FCs (Fig. S7).

We conclude that, during oogenesis, EcR signalling is required for the formation of microvilli in the apical domain of FCs. While block of EcR function from stage 7 onward does not alter the basal domain of FCs.


A
Act>>EcRB2-DN (CD2 -)
Lectin CD2
CD2
Lectin
Lectin CD2
B
Cy2>EcRB2-DN
F-actin Lectin
F-actin
Lectin
F-actin Lectin
C
Cy2>+
F-actin Lectin
F-actin
Lectin
F-actin Lectin
D
Act>>EcRB2-DN (mGFP +)
Cad99C mGFP Yl
mGFP Yl
Cad99CYl
Cad99C mGFP Yl
E
Cy2>EcRB2-DN (nGFP +)
F-actin Yl nGFP
F-actin nGFP
Yl nGFP
F-actin Yl nGFP
F
Cy2>+ (nGFP +)
F-actin Yl nGFP
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Yl nGFP
F-actin Yl nGFP
G
Act>>EcRB2-DN (CD2 -)
Ph F-actin CD2
Ph CD2
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◀**Fig. 6** Blocking EcR signalling alters the organisation of the oocyte membrane. The organisation of **a**–**g** from *left* to *right* is the same as in Fig. 4. Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; +/+; UAS-EcRB2-DN/+* and stained with anti-CD2 ab (*cyan*) and Lectin dye (*green*) (**a**). The FC flp-out clone expressing EcRB2-DN is indicated by the absence of CD2 and is marked by the *brackets*. Confocal microscopy analysis of egg chambers dissected from *w/w*; *Cy2-Gal4/+; tub-Gal80^{ts}/UAS-EcRB2-DN* (**b**) and from *w/w*; *Cy2-Gal4/+; tub-Gal80^{ts}/+* (**c**) females and stained with the Lectin dye (*green*) and phalloidin (*red* in **b** and *cyan* in **c**). The *asterisks* in **b** indicate protrusions of the actin cytoskeleton of the oocyte. Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; UAS-mGFP/+; UAS-EcRB2-DN/+* female stained with anti-Cad99C (*cyan*) and anti-Yl (*red*) abs (**d**). The FC flp-out clone expressing EcRB2-DN is indicated by the GFP expression (*green*) and is marked by the *brackets*. Confocal microscopy analysis of egg chambers dissected from *w/w; Cy2-Gal4/UAS-nGFP; tub-Gal80^{ts}/UAS-EcRB2-DN* (**e**) and from *w/w; Cy2-Gal4/UAS-nGFP; tub-Gal80^{ts}/+* (**f**) females stained with phalloidin (*cyan*) and anti-Yl ab (*red*). Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; Flu-ph/+; UAS-EcRB2-DN/+* female stained with phalloidin (*red*), anti-CD2 ab (*green*), and anti-HA ab (*cyan*) (**g**). The absence of the CD2 indicates FCs expressing EcRB2-DN (*brackets*). Anterior is *up* in **a**, **c**, **d** and on the *left* in **b**, **e**–**g**. *Scale bars* **a**–**g**, *left* 50 μm and **a**–**g** magnifications 5 μm

Altered EcR signalling in follicle cells perturbs the organisation of the oocyte membrane

The alteration of EcR signalling triggers modifications of the FC membrane and of the F-actin cytoskeleton. Thus, we decided to study the oocyte membrane to find out whether the altered architecture of oocyte cortical actin is due to abnormalities of the oocyte membrane itself or to a detachment of actin from the same membrane.

The glycoproteins of the oocyte plasma membrane are preferentially stained with fluorescein-*Lycopersicon esculentum* lectin [45]. The regions of the oocyte membrane facing the EcRB2-DN-positive flp-out FC clones are thinner than the flanking regions, facing the wild-type FCs (Fig. 6a). The alterations of the oocyte plasma membrane are magnified when EcRB2-DN is expressed in all cells of the follicular epithelium, under the control of the *Cy2-Gal4* driver. In this case the oocyte membrane, indicated by the lectin staining, enters the oocyte cytoplasm (Fig. 6b) in contrast to the oocyte membrane of control egg chambers (Fig. 6c). The double staining with phalloidin and lectin shows round protrusions into the oocyte cytoplasm generated by the oocyte membrane and the actin cytoskeleton together, with identical pattern, suggesting tight contact between the two molecules.

To further analyse the structure of the oocyte plasma membrane, we checked the distribution of the Yolkless (Yl) protein. This transmembrane receptor mediates the endocytic uptake of yolk proteins and, at stage 8, at the beginning of vitellogenesis, is transported from the ooplasm to the oocyte membrane [46]. As oogenesis continues, Yl accumulates gradually, and by stage 10 it is localised exclusively in the oocyte membrane. The comparison between the EcRB2-DN-positive flp-out FC clones and the flanking wild-type FCs shows that the Yl protein is more compact and strictly connected to the underlying microvilli of the follicle cell membrane (Fig. 6d). The results are confirmed by the experiment in Fig. 6e where EcR signalling is knocked down under the control of the *Cy2-Gal4* driver. In contrast to control egg chambers (Fig. 6f), the transmembrane receptor Yl and the F-actin form distinct round structures with identical distribution, which invade the oocyte cytoplasm.

We envisaged the possibility that the oocyte membrane damage, observed so far, may alter the transition of proteins secreted from the oocyte to the external surface. Thus, we decided to study the Polehole (Ph) protein which is secreted on the surface of the oocyte [47]. The results show that, in the region of the oocyte membrane facing the EcRB2-DN-positive flp-out clones, the secretion of the Flu-tagged-Ph protein occurs but the distribution of the protein on the oocyte surface is discontinuous and disorderly (Fig. 6g).

We conclude that the altered EcR signalling in FCs causes structural changes of the apical membrane, coupled with disorganisation of the membrane structure in the underlying oocyte. This suggests a crosstalk between the FCs and the oocyte, essential to build the correct architecture of the oocyte membrane.

Negative control of EcR: the block of EcR signalling drives Moesin activation

Moe plays a crucial role in crosslinking membrane proteins to the actin cytoskeleton [18]. It is possible that our results are due to the impairment of Moe function causing alterations of the apical domain of EcRB2-DN-positive FCs. Phosphorylation of Moe in wing disc cells relies on activity of the Slik kinase [48]. Furthermore, in FCs at stage 13 of oogenesis, Moe activation requires Sip1, and it has been suggested that Sip1 controls Moe activation by governing Slik localisation [49]. We have analysed the activation of Moe and the expression of the Sip1 and Slik proteins in EcRB2-DN-positive flp-out clones where the phosphorylation of the protein is detected using Moe phospho-specific ab (anti-pMoe) [50]. Figure 7a shows an increased phosphorylation of Moe as well as greatly enhanced levels of Sip1. Figure 7b shows that also the Slik kinase expression is increased when compared to the amount observed in the neighbouring wild-type FCs. This observation suggests a negative control of the EcR signal on the activation of the Moe protein and on the amount of the Sip1 and Slik proteins.

To find out whether a correlation exists between enhanced aPKC level and pMoe, Sip1 and Slik increased levels we have carried out the same analysis as above in flp-out clones of FCs expressing the *UAS-aPKC*^{CA} transgene. The results show a direct effect of aPKC on the quantity of the Sip1 protein that is elevated (Fig. 7c) while Moe phosphorylation and the Slik protein are not increased (Fig. 7d). In summary, it appears that enhancement of aPKC levels per se cannot lead to higher level of pMoe. Furthermore, Sip1 increase does not lead to enhanced pMoe suggesting that Slik is limiting. Alternatively, at stage 10B a different kinase–scaffold complex could act.

We have shown that EcR signalling regulates a number of molecules involved in the activation of the Moe protein. Then, we have expressed the Moe^{T559D}-Myc protein that mimics Moe constitutive activation (Moe^{CA}) [50], in flp-out clones of FCs. In Fig. S8, the staining with anti-Myc ab shows that the tagged protein is very abundant in these cells. We checked the position of the FC clones expressing Moe^{CA} in egg chambers at stage 10B and found that 100 % of the clones were in the correct place ($n = 24$). The same egg chambers were stained with Cad99C ab and phalloidin. Figure 7e shows that the FC flp-out clones expressing Moe^{CA} contain microvilli more tightly packed and longer than the neighbouring wild-type cells. In addition, the spiky pattern of the F-actin cytoskeleton between FCs and oocyte is slightly more evident than what we have observed in the wild-type flanking cells. These results indicate that the expression of a constitutively active Moe protein modifies the assembly of microvilli in FCs.

Tramtrack 69 function in FCs is required for morphogenesis of microvilli

At stage 10B, EcR signalling in FCs positively controls the exit from the endocycle by upregulating the expression of the transcription factor isoform Tramtrack 69 (Ttk69) [28]. Thus, EcR-DN expression downregulates Ttk69, delays endocycle and causes reduced cell size [28, 29]. Our results on EcR signalling show that it is required in FCs at stage 10B also for correct microvilli morphogenesis. To investigate the role of Ttk69 in microvilli formation, the phenotypic effect of its loss of function has been analysed. Using MARCM (Mosaic Analysis with a Repressible Cell Marker) [32] technique, we have obtained FC clones homozygous for the *ttk*^{1e11} null allele [30] and analysed microvilli formation. Cad99C and phalloidin stainings of mosaic stage 10 egg chambers show that *ttk*^{1e11} FCs, marked by nGFP expression, have flattened microvilli and that actin does not form the core spikes of microvilli (Fig. 8a). Furthermore, the staining of aPKC shows enhanced level in the apical region of mutant FCs (Fig. 8b). We also checked the position of *ttk*^{1e11} FC clones at stage 10B and found that they are incorrectly localised (Fig. 8c).

Discussion

Our results on the targeted block and rescue of EcR signalling in FC clones show that the EcR isoforms act redundantly in controlling growth and positioning of FCs, since efficient suppression of the EcR-DN-induced phenotype occurred irrespective of the isoform co-expressed. Redundancy has also been described in other tissues [24] and could provide functional stability and strength against component failure.

Morphogenesis of the follicular epithelium requires multiple differentiation events through which FCs acquire specific cell fates and become competent to perform a number of activities [8]. At stage 9 of oogenesis, the border cells start to migrate through the nurse cells to reach the anterior border of the oocyte. Ecdysone signalling controls the timing of this migration [39]. At the same stage, the remaining FCs are progressively displaced toward the oocyte and by stage 10A few squamous FCs cover the nurse cells while the vast majority of FCs became columnar and overlay the oocyte. Although the oocyte and the entire egg chamber grow, the distribution of squamous and columnar FCs is maintained in stage 10B egg chamber. Interestingly, the block of EcR signalling in clones of FCs halts the correct localisation of the main body FCs (this paper and [29]). Furthermore, we uncover a broader requirement of EcR signalling since expression of EcR-DN from stage 7 of oogenesis in FCs prevents main body FCs to follow the oocyte growth. The inability of main body FCs to cover the oocyte may be due to their reduced cell size as well as to loss of adhesion between columnar FCs and the oocyte. We favour the latter hypothesis since reduced FC size due to silencing of the Myc growth factor in FC clones does not impair correct positioning of FCs (not shown). In addition, we found that mispositioning of FCs induced by blocking EcR signalling from stage 7 is coupled to defects in the oocyte membrane. Staining of glycoprotein, transmembrane receptor Yl and actin components outlines infolding of the plasma membrane in the region facing EcR-DN mutant FCs. Therefore, we suggest that EcR signalling is required in FCs for establishment of correct adhesion with the underlying germline and that this in turn would permit correct positioning of main body FCs as well as appropriate organisation of oocyte membrane. Furthermore, the rescue of FC positioning due to silencing of the aPKC apical determinant in FCs lacking EcR signalling indicates the importance of correct establishment of the apical domain. Thus, this domain could contain molecular components relevant for cell adhesion whose

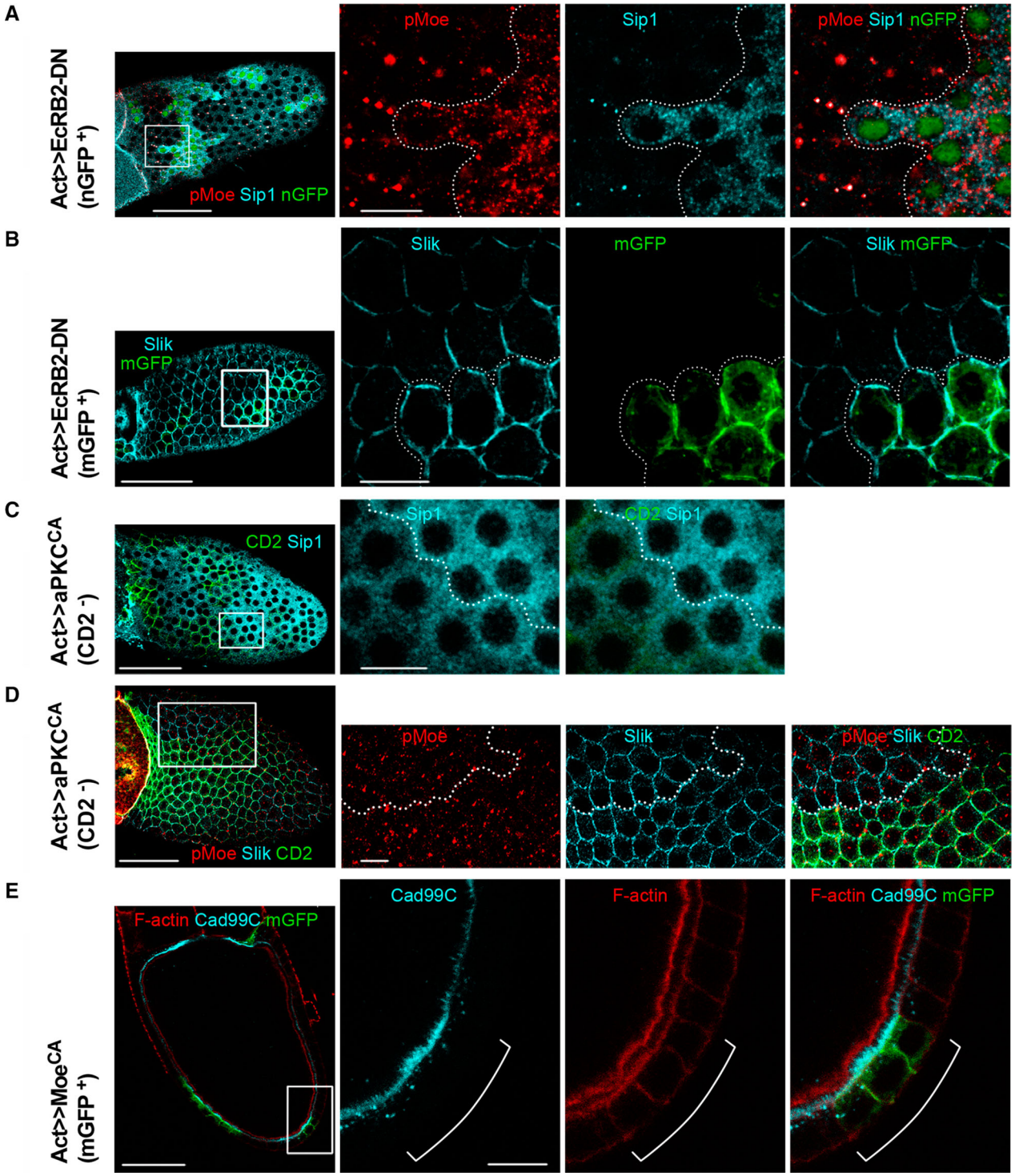

Fig. 7 Blocking EcR signalling enhances levels of pMoe. The FC flp-out clones are marked by the expression of the GFP protein (*green*) (**a**, **b**, **e**) and by the absence of the CD2 marker (**c**, **d**). Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; UAS-nGFP/+; UAS-EcRB2-DN/+* stained with anti-pMoe (*red*) and anti-Sip1 (*cyan*) ab (**a**). Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; UAS-mGFP/+; UAS-EcRB2-DN/+* stained with anti-Slik ab (*cyan*) (**b**). Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; UAS-aPKC^{CA}/+; +/+* (**c**, **d**) stained with anti-CD2 (*green* in **c**, **d**), anti-Sip1 (*cyan* in **c**) and anti-pMoe (*red*) and anti-Slik (*cyan*) in **d**. Confocal microscopy analysis of a stage 10B egg chamber dissected from *hs-flp/Act>>Gal4; UAS-mGFP/UAS-Moe^{CA}-Myc* females stained with anti-Cad99C ab (*cyan*) and phalloidin (*red*) (**e**). The *dotted lines* in **a**–**d** mark the boundary between the wild-type and EcRB2-DN expressing cells (**a**, **b**) and between the wild-type and aPKC^{CA} expressing cells (**c**, **d**). Anterior is on the *left* in *panels* **a**–**d**, and *up* in **e**. *Scale bars* **a**–**e** *left* 50 μm and **a**–**e** magnifications 10 μm

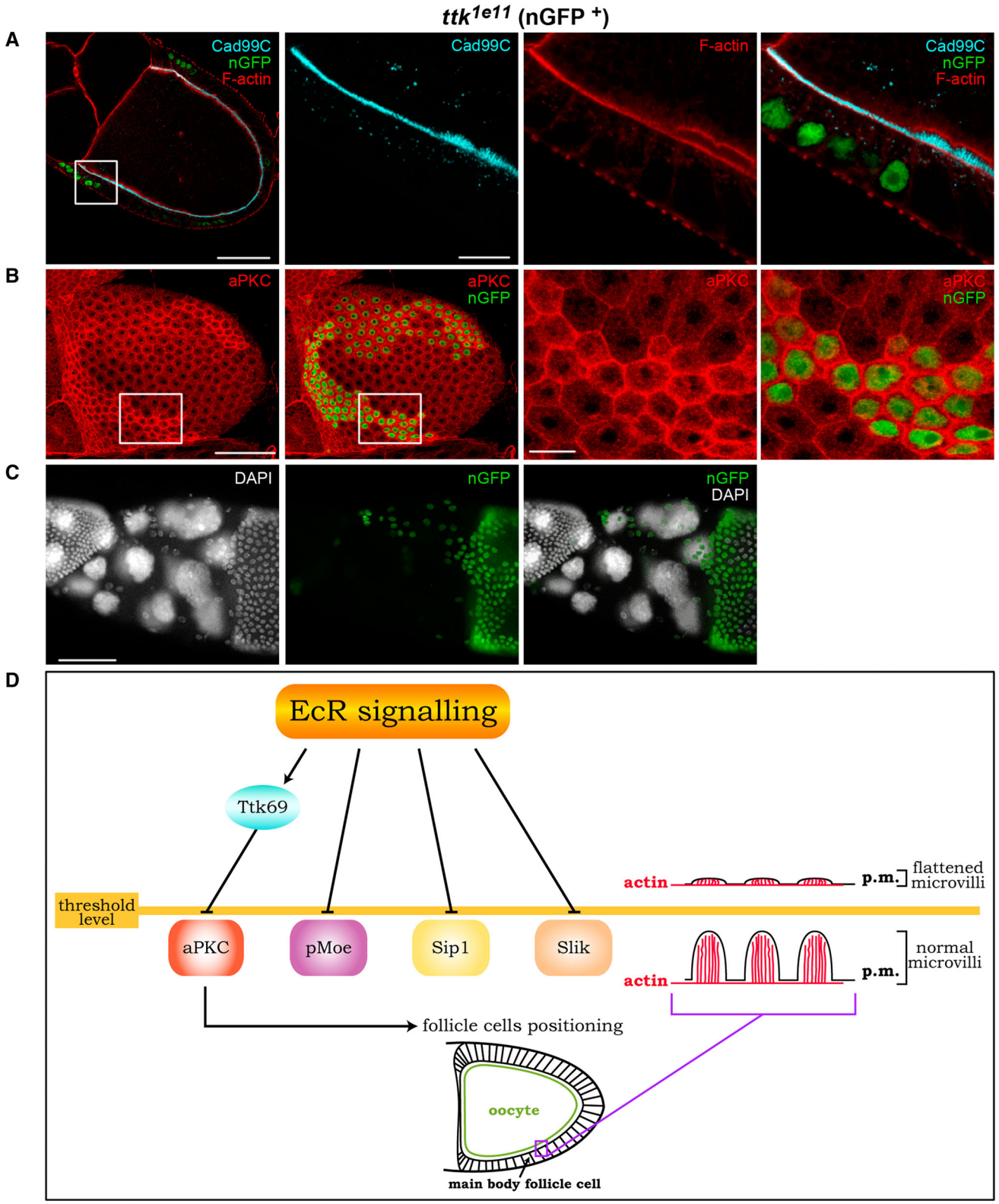

Fig. 8 Morphogenesis of microvilli is altered in *ttk*^{1e11} loss of function clones. Confocal microscopy analysis (**a**, **b**) and fluorescence microscopy analysis (**c**) of stage 10B egg chambers dissected from *hs-flp, tub-Gal4, UAS-nGFP/+*; *+/+*; *FRT82B, tub-Gal80/FRT82B, ttk*^{1e11} stained with phalloidin (*red*) and anti-Cad99C ab (*cyan* in **a**), anti-aPKC ab (*red* in **b**) and DAPI (*white* in **c**). The *ttk*^{1e11} FC clones are indicated by the presence of nGFP (*green* in **a**–**c**). Scheme of the EcR signalling targets. *p.m.* plasma membrane (**d**). Anterior is *up* in **a** and on the *left* in **b**, **c**. *Scale bars* **a**–**c** *left panels* 50 µm and **a**, **b** magnifications 10 µm

distribution could be altered, directly or indirectly, by enhanced aPKC levels. It has been reported that intercellular communication between germline and somatic FCs mediated by gap junctions plays a key role in the process of egg chamber formation [51]. Moreover, gap junctions mediate stage-specific bioelectric signals in different population of FCs at mid-oogenesis and it has been proposed that these signals play a role in morphogenetic processes that occur during this stage including cell migration and thus cell positioning [52].

Our analysis shows for the first time that EcR signalling positively regulates the morphogenesis of microvilli. In wild-type egg chambers, these apical protrusions elongate from FC membrane at stage 10 and shorten during following stage 11. Microvilli fulfil a key role in the assembly of vitelline membrane [16, 17], the innermost eggshell layer that stores the positional information required for embryonic patterning [53]. Impaired assembly of vitelline membrane, due to loss or disorganisation of FC microvilli, in turn alters functional properties of the eggshell so that embryonic development stops causing female sterility [16, 54]. We found a functional requirement for aPKC on the normal growth of apical microvilli, as the constitutive activation of aPKC flattens them, while silencing of aPKC increases their length. Interestingly, aPKC seems to act mainly at level of the membrane, as alterations of aPKC affect only mildly the microvilli actin structure. Since downregulation of aPKC in FCs without EcR signalling does not rescue the flattening of microvilli, we suggest the presence of additional effector(s) controlling microvilli morphogenesis.

A further control of EcR signalling in FCs is the activation of Moe, which plays a role in the coordination of border cell migration [55] and in the morphogenesis of microvilli in rhabdomeres [50]. We also found that EcR signalling controls level of Slik and Sip1 proteins whose involvement in Moe phosphorylation at late stage of oogenesis has been shown [49]. The increased level of Moe phosphorylation in FCs correlates with altered morphogenesis of the microvilli. Accordingly, the expression of the constitutively active Moe protein leads to altered microvilli and does not appear to affect cortical actin suggesting a role of Moe in the construction and maintenance of correct apical domain of FCs. In conclusion, our results show that EcR signalling cascade negatively controls aPKC and pMoe to maintain these key cellular components at a level required for microvilli morphogenesis (Fig. 8d).

Our analysis also shows that the Ttk69 transcription factor positively controls morphogenesis of microvilli at stage 10 as well as positioning of main body FCs. Upregulation of Ttk69, driven by EcR signalling at stage 10, plays key roles in controlling exit from the endocycle [28] and apical size of FCs [29]. Indeed, loss of Ttk69 function in main body FCs causes the apical constriction that in wild-type egg chambers occurs only in the two groups of dorsal-appendage-forming FCs, at late stage 10 [29]. Our present result fits well with the role of EcR signalling and Ttk69 in controlling apical domain of FCs and show that microvilli formation is a so far undescribed differentiation event mediated by EcR signalling at stage 10.

Interestingly, a role for transcriptional control of EcR signalling has been demonstrated for a vitelline membrane structural gene [25]. This positive control is accomplished through the Ttk69 transcription factor activity [56]. In summary, EcR signalling in FCs at stage 10B simultaneously controls both expression of structural components of vitelline membrane and formation of the apical membrane microvilli required for correct assembly of this envelope.

In conclusion, this study shows that EcR signalling controls an intricate molecular network to allow the correct morphogenesis of both the follicular epithelium and the egg chamber. Unravelling this effector network will require additional effort and will lead to a better understanding of the molecular mechanisms acting in differentiation processes.

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