Rapid apical actin filament turnover is required for non-centrosomal microtubule organization in epithelium

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ABSTRACT

Epithelial cells rely on the precise coordination of actin and microtubule cytoskeletons to maintain their polarized structure and function. Microtubules form longitudinal non-centrosomal arrays that are essential for organelle positioning and facilitate the directional trafficking of cargoes within polarized epithelial cells. The apical actin cytoskeleton is crucial for supporting cell shape and overall tissue architecture. This study investigates the interplay between apical actin dynamics and microtubule organization *in vivo* within *Drosophila* epithelial tissue. We show that the loss of Cyclase-Associated Protein (CAP) leads to the excessive accumulation of unusually stable actin structures at the apical cortex, which subsequently exclude microtubules and membrane-bound vesicles. In CAP mutant cells, the apical localization of microtubule minus-end organizing proteins is impaired, emphasizing the role of actin dynamics in microtubule anchoring. Consequently, non-centrosomal microtubule-dependent processes are impaired, leading to nuclear mispositioning, disrupted apical cargo transport, and ultimately defective microvilli formation in CAP mutant cells. Our findings highlight that rapid apical actin turnover, catalyzed by CAP, is critical for the organization of non-centrosomal microtubules. This study emphasizes the intricate crosstalk between actin filaments and microtubules, and how crosstalk between actin filaments and microtubules sustains the polarized functions of epithelial tissues.

INTRODUCTION

Epithelia are sheets of polarized cells that line tissues and serve as protective barriers against the external environment. These cells exhibit distinct apical and basal surfaces, which enables key epithelial functions such as barrier function, absorption, secretion, and transport (Buckley & St Johnston, 2022). The establishment and maintenance of epithelial cell structure and function depend on the coordinated organization of the actin and microtubule cytoskeletons (Raman *et al.,* 2018). While the roles of microtubules in supporting polarized functions are well established (Meiring *et al.,* 2020), the dynamic interactions between actin and microtubules at the apical cortex remain less understood.

In most epithelia, microtubules are organized into non-centrosomal arrays with their minus-ends anchored at the apical cortex and plus-ends extending toward the basal side (Mogensen & Tucker, 1987; Meads & Schroer, 1995; Bartolini & Gundersen, 2006; Toya & Takeichi, 2016; Perrin & Matic Vignjevic, 2023). This polarized arrangement supports directional trafficking by dynein and kinesin motor proteins, enabling the targeted delivery of vesicles and organelles to apical and basolateral membrane domains, respectively (Reck-Peterson *et al.,* 2018, Kreitzer & Myat, 2018). The establishment of the non-centrosomal microtubule arrays depends on the apical anchoring of microtubule minus-ends, a process that requires coordinated action of the microtubule-associated proteins CAMSAP3/ *Drosophila* Patronin, and ACF7/MACF1/ *Drosophila* spectraplakin Shot, as well as the apical spectrin cytoskeleton (Khanal *et al.,* 2016; Nashchekin *et al.,* 2016; Ning *et al.,* 2016; Noordstra *et al.,* 2016; Toya *et al.,* 2016; Muroyama *et al.,* 2018;). CAMSAP3/Patronin binds, stabilizes, and anchors the minus ends of microtubules at the apical cortex (Goodwin & Vale, 2010; Toya *et al.,* 2016). Spectraplakin Shot is an actin-microtubule crosslinker and sole *Drosophila* homolog on human dystonin and ACF7 (MACF1). Shot crosslinks microtubules to the actin cytoskeleton, aiding in the assembly and maintenance of non-centrosomal microtubule arrays (Lee & Kolodziej, 2002; Applewhite *et al.,* 2010).

The actin cytoskeleton, in turn, forms a dense apical network essential for maintaining epithelial cell shape, tissue architecture, and mechanical integrity (Pelaseyed & Bretscher, 2018). Apical actin also supports vesicle trafficking, junctional organization, and cell–cell adhesion. Many epithelial cells also feature actin-rich apical structures such as microvilli or stereocilia, that are crucial for epithelial tissue functions like nutrient absorption and sensory perception (Sauvanet *et al.,* 2015; Sharkova *et al.,* 2023). The biogenesis of apical microvilli relies on proper delivery of membrane and cargo (such as microvilli-specific cadherin PCDH15 / *Drosophila* Cad99C) via dynein- and Rab11-mediated transport along non-centrosomal

microtubules. Defects in this process disrupt microvillar formation (D'Alterio *et al.,* 2005; Knowles *et al.,* 2014; Khanal *et al.,* 2016;). Moreover, apical, or junctional actomyosin networks, comprising of contractile actin filaments and myosin-2, are key forces driving epithelial morphogenesis (Martin *et al.,* 2009; Roh-Johnson *et al.,* 2012).

It has become increasingly clear that actin and microtubule cytoskeletons interact and are coordinated (Pimm & Henty-Ridilla, 2021), which is recognized as especially important for the regulation of cell shape and polarity in migrating cells, as well as neuronal and epithelial cells (Dogterom & Koenderink, 2019). Studies conducted *in vitro* and in cultured cells have revealed that actin structures actively modulate microtubule dynamics and organization over time and space. In particular, the organization and density of filamentous actin not only supports cellular architecture but also has direct consequences for microtubule dynamics and organization, for instance at leading edge of migrating cells or regulating centrosomal microtubule nucleation during mitosis (Waterman-Storer & Salmon, 1997; Ballestrem *et al.,* 2000; Gupton *et al.,* 2002; Gupton & Waterman-Storer, 2006; Colin *et al.,* 2018; Farina *et al.,* 2019; Inoue *et al.,* 2019; Gélin *et al.,* 2023). However, it remains less explored how actin dynamic affects structural organization of actin and actin-microtubule coordination, especially in the context of epithelial tissue *in vivo*.

In this study, we utilize the *Drosophila* follicular epithelium as a model system to investigate the critical role of actin dynamics in the organization and function of microtubules. Our study demonstrates that cyclase-associated protein (CAP) -mediated apical actin turnover is essential for the establishment and organization of non-centrosomal microtubule arrays in the *Drosophila* follicular epithelium. We show that actin accumulation in CAP deficient cells leads to disorganization of non-centrosomal microtubule arrays, causing mispositioning of cell nuclei and impairment of dynein-based apical cargo trafficking, ultimately affecting microvilli biogenesis. Together, these findings reveal the apical cortex of epithelial cells as a key site for actin-microtubule coordination, highlighting the importance of actin dynamics for establishment and maintenance of non-centrosomal microtubules and supporting polarized function and structure of epithelial cells.

RESULTS

Excess apical actin impairs microtubule organization in epithelial cells *in vivo*

To investigate the role of apical actin turnover in epithelial cells, we focused on Cyclase-associated protein (CAP), a key regulator of actin dynamics known for its ability to promote the depolymerization of cofilin-decorated actin filaments and subsequently re-charging the resulting actin monomers with ATP (Ono *et al.,* 2013). Notably, loss of CAP leads to aa excessive accumulation of filamentous actin specifically at the apical side (Baum

& Perrimon, 2001) of epithelial cells of *Drosophila* follicular epithelium (Figure 1 A; Supl. Figure 1 A), a striking phenotype given the polarized architecture and function of these cells. Despite highly localized accumulated actin, CAP protein itself displayed mostly diffuse cytoplasmic localization (Supl. Figure 1 A). Apical accumulation of F-actin suggests that in follicular epithelium CAP targets specific actin filament populations undergoing rapid turnover, as has been reported in other systems (Bertling *et al.,* 2004; Ono, 2013). Thus, we investigated if specific actin filament configurations would be enriched in CAP mutant cells. Indeed, accumulated apical actin in CAP mutant cells displayed increased levels of proteins promoting rapid actin filament disassembly, such as Twinfilin and Aip1 (Supl. Figure 1 B, D), suggesting compensatory mechanisms for managing excess of filamentous actin. While the actin filament elongator Ena was enriched in these actin structures (Supl. Figure 1 E; Baum & Perrimon, 2001), profilin, which supplies monomeric actin for elongation, was expressed at higher level but missing from the site of actin accumulation (Supl. Figure 1 C), indicating that an excess of actin filament assembly is unlikely to occur at the apical side of CAP mutant cells. Additionally, tropomyosin and α-actinin were enriched at the site of actin accumulation (Supl. Figure 1 F-G), whereas active non-muscle myosin II was either partially or completely excluded from apical actin networks in a stage-dependent manner, demonstrating that the accumulated actin is unlikely to form contractile actomyosin filaments (Supl. Figure 1 H, H'). In conclusion, the dense and stabilized actin in CAP mutant cells is composed of a complex mixture of filamentous actin and various actin-binding proteins but appears to lack contractile properties.

Notably, dense actin structures can act as barriers, preventing and/or guiding the growth of microtubules *in vitro* and in cultured cells (Gupton & Waterman-Storer, 2006; Colin *et al.,* 2018; Gélin *et al.,* 2023). Therefore, we characterized organization of microtubules in CAP mutant tissues. Interestingly, the apical region of CAP mutant cells, characterized by accumulated actin, exhibited decreased levels of stable acetylated microtubules (Figure 1 B-D) as well as α- and β-tubulin (Figure 1 , E, F), indicating that microtubules were excluded from the region of actin accumulation. We then analyzed microtubule organization in stretch cells which are specialized flattened follicle cells. The morphology of stretch cells facilitates visualization of the cytoskeleton within the whole cell. In CAP mutant stretch cells, actin accumulation was predominant in one cytoplasmic region, where stable acetylated microtubules did not penetrate (Figure 1 G). Together, these findings reveal that dense actin structures exclude microtubules in epithelial cells *in vivo*.

Elevated levels of apical actin affect localization of microtubule-actin crosslinking protein

spectraplakin/Shot, involved in non-centrosomal microtubule organization

In many epithelial cells, microtubules are organized in a non-centrosomal array, with their minus-ends anchored to the apical cortex and plus ends extending basally (Bacallao *et al.,* 1989; Bré *et al.,* 1990; Clark *et al.,* 1997). Thus, we aimed to determine whether apical actin accumulation affects the microtubule minus end anchoring, mediated by Patronin and Shot, and apical spectrins. To this end, we detected Patronin, Shot, and α-spectrin in CAP mutant cells (Khanal *et al.,* 2016). We found that in CAP mutant cells, Patronin was still localized apically, but in a more diffuse manner as compared to the sharp apical localization observed in wild type cells (Figure 2 A). In addition, the localization of Shot in the CAP mutant cells was found enriched around the accumulated actin compared to the wild type cells (Figure 2 B). Finally, the apical localization of α-spectrin was partially lost (Figure 2 C). Taken together, our results demonstrate that apical actin turnover is required for the proper localization of Patronin, Shot and α-spectrin in epithelial cells.

Apical actin dynamics is essential for microtubule organization to ensure correct nuclear positioning

Organization of the non-centrosomal microtubules is required for proper nuclear positioning in various cellular settings such as in fly follicular epithelium as well as in mammalian intestinal epithelial cells (Khanal *et al.,* 2016; Nashchekin *et al.,* 2016; Toya *et al.,* 2016; Muroyama *et al.,* 2018). We found that in CAP mutant cells, nuclei were mispositioned toward the apical region compared to the basal positions of the neighboring control cells (Figure 3 A). Quantitative analysis confirmed that the distance of the nuclei from the basal membrane was significantly greater in CAP mutants as compared to the controls (Figure 3 C). These results indicate that an excess of apical actin affects nuclear positioning in follicular epithelium. Surprisingly, reintroducing wild-type CAP into CAP mutant cells did not restore nuclear positioning, despite successfully rescuing actin accumulation and microtubule organization (Figure 3 B, C). It is important to note that while we quantified nuclear positioning during stages 9-10, positioning occurs earlier, during stages 6-9 of oogenesis (Szikora *et al.,* 2013). Thus, we asked if the CAP mutant phenotypes are visible at earlier stages of the follicle development. Indeed, the loss of CAP led to actin accumulation and disrupted apical microtubule organization in follicle cells during both early (stage 7, Figure 3 D) and late (stage 10, Figure 3 E) stages of oogenesis. However, the reintroduction of wild-type CAP only rescued actin accumulation and microtubule organization at stage 10, not at stage 7 (Figure 3 F, G). Further analysis showed that the CAP rescue construct was only minimally expressed during stages 6-7 (Figure 3 H) indicating that the nuclear mispositioning phenotype, which

develops during the early stages of oogenesis, could not be rescued by CAP expression at later stages. In conclusion, these results underscore the temporal requirement of actin dynamics for establishing non-centrosomal microtubule arrays for ensuring correct nuclear positioning.

Disruption of actin dynamics results in abnormal distribution of membrane-bound organelles

Given that the dense actin structures in CAP mutant cells exclude microtubules, we next asked if vesicular cargoes trafficked via microtubules are also affected. Here, we used mCD8-GFP as a clonal marker to outline the mutant cells in chimeric tissues (Lee & Luo, 2001). The membrane-bound mCD8-GFP labels cell membranes and apical microvilli (Blanc *et al.,* 1988; Burr *et al.,* 2014). In follicle cells, mCD8-GFP marks also the entire cytoplasm, possibly highlighting the enrichment of endomembrane compartments in these cells. Interestingly, in CAP mutant cells mCD8-GFP-labeled compartments were lost at the regions of actin accumulation. The phenotype was rescued by reintroducing wild-type CAP (Figure 4 A, B). This result suggested a significant impairment in the normal distribution of endomembrane components in CAP mutant cells. Indeed, the site of actin accumulation was devoid of membrane-bound organelles, as indicated by the absence of specific markers for the endoplasmic reticulum (ER marker calnexin Cnx99A) and Golgi (trans-Golgi-localizing Golgin 84) (Figure 4 C, D). Transmission electron microscopy (TEM) imaging of CAP mutant cells confirmed that the site of actin accumulation lacked all membrane-bound cytoplasmic components (Figure 4 E i, ii). Additionally, CAP mutant cells displayed ectopic electron-dense vesicular structures both beneath and surrounding the site of actin accumulation (Figure 4 E iii, iv). The intensity of staining resembles that of the perivitelline space and—suggests that these vesicular structures contain perivitelline components destined for apical secretion. The accumulation of the vesicular structures strongly suggests a disruption in the normal trafficking pathways in the CAP mutant cells. In conclusion, our results demonstrate the critical role of the actin turnover in maintaining proper organization and dispersal of membrane-bound vesicles and organelles.

Apical actin turnover enables efficient dynein-mediated transport of Rab11 endosomes, essential for microvilli formation

Next, we investigated whether apical actin turnover affects microtubule-based transport *in vivo*. Proper organization of non-centrosomal microtubules is crucial for maintaining intracellular architecture, directional trafficking, and microvilli biogenesis (Khanal *et al.,* 2016; Nashchekin *et al.,* 2016; Noordstra *et al.,* 2016; Toya *et al.,* 2016; Muroyama *et al.,* 201). Transport of apical cargoes is mediated by the microtubule minus-end-directed motor protein dynein (Wilkie & Davis, 2001; Harris & Peifer, 2005; Horne-Badovinac & Bilder, 2008; Reck-

Peterson *et al.,* 2018), which carries Rab11-positive recycling endosomes toward the apical cortex, where microtubule minus-ends are anchored. Rab11, a member of the RAB GTPase family, is critical for polarized trafficking of various cargoes, including membrane proteins and junctional components (Casanova *et al.,* 1999; Jing & Prekeris, 2009; Roeth *et al.,* 2009). Thus, we investigated whether microtubule-based transport is disrupted in CAP mutant cells. We first examined the localization of Dynein and Rab11-positive endosomes in the CAP mutant follicle cells. In control cells, both Dynein and Rab11 were enriched at the apical surface. However, in CAP mutant cells, Dynein and Rab11 localized beneath the accumulated apical actin (Figure 5 A–B, D–G). As Dynein marks the minus-ends of microtubules, their localization in CAP mutant cells shows that polarity of non-centrosomal microtubules is intact. However, the localization of Rab11 beneath accumulated actin suggest that dynein-mediated minus-end-directed vesicle transport is halted in CAP mutant follicle cells by the excess apical actin.

To further verify the fate of apical cargoes, we performed immunostaining of protocadherin Cad99C (D'Alterio *et al.,* 2005; Schlichting *et al.,* 2005), an established cargo transported by Dynein and Rab11 in follicle cells (Khanal *et al.,* 2016). In wild-type cells, Cad99C was localized at the apical microvilli (Figure 5 C). However, in CAP mutant cells, the localization of Cad99C at the apical surface was reduced, with most of the protein retaining beneath the site of actin accumulation, similarly to Dynein and Rab11 (Figure 5 H-I). This result further demonstrated that the dense apical actin networks in CAP mutant cells prevent Dynein-mediated trafficking of Cad99C-containing Rab11-positive vesicles to the apical surface. Since Cad99C is also crucial for microvilli biogenesis, we measured the length of apical microvilli and found them to be significantly shorter in CAP mutant cells compared to controls or mutant cells rescued with wild type CAP (Figure 5 J-L). Taken together, these results demonstrate the necessity of apical actin turnover for Dynein-mediated trafficking of apical cargoes and for microvilli biogenesis.

Nucleotide exchange activity of CAP is crucial for maintaining proper apical actin dynamics

Next, we wanted to understand how CAP mediates apical actin turnover *in vivo*. Existing biochemical, structural, and cell biological studies have contributed to a comprehensive understanding of how CAP's biochemical activities are orchestrated to promote actin turnover (Figure 6 A). Therefore, we generated mutant constructs targeting critical amino acids in the HFD, PP, WH2, and CARP domains (Figure 6 A, Table 1). We then tested the ability of these constructs to rescue the CAP mutant phenotype *in vivo*. Our results show that CAP constructs with mutations in HFD, PP, and WH2 domains rescued the actin accumulation phenotype in stages 8-10 of oogenesis (Figure 6 C-D, Supl Figure 2 C-E). The CARP domain mutant did not

rescue actin accumulation in the CAP mutant cells even in stages 9-10. This result show that the nucleotide exchange activity of CAP is critical for efficient apical actin turnover (Figure 6 D). This result aligns with the fact that nucleotide exchange is the most conserved function of CAP across eukaryotes (Ono, 2013) its loss leads to significant actin accumulation and phenotype changes in yeast (Kotila *et al.,* 2018). Another established nucleotide exchange factor for actin is profilin, and in budding yeast the loss of CAP can be rescued by over-expression of profilin (Vojtek *et al.,* 1991). However, in CAP mutant follicle cells over-expression of profilin did not rescue apical actin accumulation (Figure 6 E). The failure of profilin overexpression to rescue the CAP mutant phenotype suggests that merely increasing nucleotide exchange is not sufficient to restore proper actin dynamics in these cells, indicating a more complex regulatory mechanism at play. Surprisingly, a rescue construct with mutations in the N-terminal domain (responsible for promoting the depolymerization of cofilin-decorated actin filaments) effectively rescued the CAP mutant phenotype. Efficient actin depolymerization typically requires multimerization mediated by the CAP N-terminus (Chaudhry *et al.,* 2013). However, *Drosophila* CAP lacks the critical N-terminal sequence for multimerization (Benlali *et al.,* 2000), which may explain this unexpected finding. In conclusion, our findings highlight the critical role of CAP's nucleotide exchange activity in regulating actin dynamics particularly at the apical region, where precise control of actin turnover is essential for maintaining non-centrosomal microtubule organization.

Stable apical actin excludes microtubules and prevents microtubule-mediated transport

The rescue data suggested that CAP plays an important role in regulating apical actin dynamics, we then wanted to investigate actin dynamics in follicular epithelium and if it is affected by the loss of CAP. Therefore, we treated the egg chambers with CAP mutant follicle clones with Latrunculin A (Lat A), which inhibits F-actin polymerization by sequestering actin monomers (Spector *et al.,* 1983). LatA can be used to monitor the stability of existing actin filaments. Latrunculin A treatment of control cells resulted in the disappearance of apical and basal actin, whereas cortical actin remained largely unaffected in stage 7 and 10 egg chambers. This suggests that apical and basal actin in follicle cells are under rapid turnover (Figure 7 A, B). In contrast, accumulated dense apical actin of CAP mutant cells persisted even after prolonged (2 h) Latrunculin A treatment (Figure 7 C). This result directly shows that accumulated apical actin filaments in the CAP mutant cells are very stable. Unexpectedly, despite strong residual filamentous actin, LatA-treatment allowed some microtubules to pass through the accumulated actin (Figure 7 C). This result suggests that the accumulated actin undergo low levels of depolymerization, leading to a decrease

in the total density following LatA-treatment to allowing microtubules to penetrate through the filamentous actin mass. Alternatively, a moderate reduction in the density of accumulated actin may have been caused by Latrunculin A itself, as it was reported to depolymerize actin filaments by severing and sequestering monomers (Fujiwara *et al.,* 2018). Furthermore, the localization of membrane-bound mCD8GFP was partially restored in the apical region of CAP mutant cells after Latrunculin A treatment (Figure 7 D). The result shows that microtubule-mediated transport of membrane-bound compartments to the apical regions is recovered by Latrunculin A treatment. Taken together, these results show that CAP loss of function leads to the formation of stable, dense apical actin structures, which forms a barrier that prevents microtubule penetration and effective microtubule-based transport.

DISCUSSION

This study demonstrates that rapid apical actin turnover mediated by Cyclase-Associated Protein (CAP, capt in *Drosophila*) is crucial for the organization and function of non-centrosomal microtubules in epithelial tissues *in vivo*. The accumulation of apical actin in CAP mutant cells disrupts non-centrosomal microtubule arrays, leading to defects in nuclear positioning, dynein-based apical cargo trafficking, and the formation of apical microvilli. This highlights CAP's critical role in mediating apical actin turnover, which is essential for cytoskeletal coordination in epithelial cells.

Cyclase associated protein (CAP) is required for correct microtubule organization

CAP, a well known actin-binding protein, promotes rapid actin turnover via its multiple biochemical function: the depolymerization of cofilin-decorated actin filaments, followed by cofilin release and the recharging of actin monomers by performing nucleotide exchange from ADP to ATP bound monomers (Figure 6 A). Our rescue analysis revealed that nucleotide exchange activity of CAP is critical for regulating apical actin levels in follicle cells.

The interplay between actin and microtubules is finely tuned by proteins that connect both cytoskeletal systems (Dogterom & Koenderink, 2018; Pimm & Henty-Ridilla, 2021). The actin-binding protein profilin facilitates assembly of actin monomers into pre-existing actin filaments, but, its loss leads to decreased actin and increased microtubule levels (Nejedla *et al.,* 2016; Henty-Ridilla *et al.,* 2017; Liu *et al.,* 2022). The microtubule phenotypes associated with profilin loss are likely indirect, resulting from adaptive responses of microtubules to changes in the actin cytoskeleton (Cisterna *et al.,* 2024). This suggests that by promoting actin filament turnover also CAP may indirectly affect microtubules. Indeed, actin filament architectures actively modulate microtubules *in vitro* and in cultured cells (Waterman-Storer & Salmon, 1997; Gupton *et al.,* 2002;

Cheng *et al.,* 2023; Gélin *et al.,* 2023). One mechanism by which filamentous actin directly regulates microtubules is through steric exclusion, where dense actin architectures can obstruct microtubule plus-end growth or cause their disassembly (Colin *et al.,* 2018). Thus, dense and persistent apical actin acts as a barrier to microtubules in CAP mutant cells. This is supported by the observation that prolonged Latrunculin A treatment "loosens" the accumulated actin in CAP mutant cells, allowing microtubules to pass through. In summary, we conclude that CAP-mediated apical actin turnover actin is essential for the spatial organization of microtubules.

Coordination of apical actin and microtubules

Our study establishes that the apical side of epithelial cells is a critical site for coordinating actin and microtubules. The interplay between actin and microtubules occurs in sites where the two cytoskeletal components converge in crowded spaces, such as at the leading edge of migrating cells or in neural growth cones (Dogterom & Koenderink, 2018; Pimm & Henty-Ridilla, 2021). In addition to regulating microtubules at the cell periphery, dense actin filaments in the centrosome reduce the number of centrosomal microtubules (Farina *et al.,* 2019; Inoue *et al.,* 2019). This suggests that dynamic changes in actin filament densities can be regulated according to the needs of microtubule assembly. Our findings indicate that CAP-mediated apical actin turnover is temporally critical during early oogenesis for establishing proper arrangements of non-centrosomal microtubule arrays.

The temporal requirement of apical actin turnover is evident from the nuclear positioning phenotype of CAP-mutant cells. In actively dividing cells, microtubules are typically nucleated by the centrosome, whereas in postmitotic epithelial cells, microtubules are organized into non-centrosomal arrays (Muroyama & Leclerc, 2017). The transition from centrosomal to non-centrosomal microtubules coincides with the termination of cell divisions. In the follicular epithelium, cells divide until stage 6 (Klusza & Deng, 2011), suggesting that transition to non-centyrosomal microtubules occurs thereafter. In follicle cells, nuclear positioning appears to be temporally connected to the establishment of non-centrosomal microtubule arrays during stages 6-9. In CAP mutant cells with stabilized apical actin, nuclei are positioned more apically compared to wild-type cells. Correct nuclear positioning in follicle cells is determined by the growing plus-ends of apically anchored microtubules that exert force to push nuclei to the correct apical-basal position (Szikora *et al.,* 2013). Thus, the nuclear positioning phenotype in CAP mutant cells is likely caused by disorganization of non-centrosomal microtubules and impaired growth of microtubule plus-ends basally. These findings suggest that apical actin turnover is critical for correct nuclear positioning at the time when non-

centrosomal microtubule arrays are established.

Both Patronin and Shot are required for organizing non-centrosomal microtubule arrays and ensuring correct nuclear positioning across various cell types, from the *Drosophila* follicular epithelium to mammalian intestinal cells (Khanal *et al.,* 2016; Nashchekin *et al.,* 2016; Toya *et al.,* 2016; Muroyama *et al.,* 2018). Normally, Shot and Patronin exhibit sharp apical localization. In CAP mutant cells, however, Patronin localization is apical but diffuse, whereas Shot is localized ectopically beneath the accumulated actin. While our current study does not reveal the mechanisms by which dense actin causes impaired localization of Shot and Patronin, existing literature provides some insights. Ectopic Shot localization in CAP mutant cells may be explained by the accumulated actin and by the fact that Shot can bind both actin and microtubules. Increased filamentous actin densities have been shown to enhance Shot's ability to bind microtubule lattices and promote actin-microtubule crosslinking (Applewhite *et al.,* 2010; Applewhite *et al.,* 2013; Takács *et al.,* 2017). It is thus possible that in CAP mutant cells, ectopically localized Shot cross-links microtubules beneath the accumulated actin, further impairing cytoskeletal coordination. The apical but diffuse localization of Patronin upon CAP loss of function, in turn, suggests that dense apical actin may hinder the diffusion of Patronin to the apical cortex. In line with this hypothesis, dense actin filaments have been shown to create diffusion barriers or act as selective filters in different cellular contexts (Song *et al.,* 2009; Balasanyan *et al.,* 2017; Cheng *et al.,* 2023). Interestingly, the apical localization of Patronin seems to be accompanied by the transition to non-centrosomal microtubule arrays . The transition to non-centrosomal microtubules involves katanin-mediated severing of centrosomal microtubules into pieces, which are then captured and stabilized by Patronin and anchored to the apical cortex of the *Drosophila* salivary gland epithelial cells (Gillard *et al.,* 2021). Thus, altered Patronin and Shot localization in CAP mutant cells may further impair non-centrosomal microtubule organization. To conclude, we propose that the apical side of epithelial cells is an important site where the actin and microtubule cytoskeletons converge, and that efficient actin turnover mediated by CAP is critical for enabling the correct organization of non-centrosomal microtubule arrays.

The coordination of apical actin and non-centrosomal microtubules has been earlier explored in the context of epithelial morphogenesis, focusing on the generation of actomyosin-mediated contractile and adhesive forces (Röper, 2020). In contrast to our findings where the actin cytoskeleton directs microtubule organization, microtubules are often described as regulators of apical actomyosin pool and coordinating actomyosin-based forces during apical constriction (Lee & Harland, 2007; Booth *et al.,* 2014; Ko *et al.,* 2019). Alongside microtubules, actin turnover is crucial for

maintaining force balance during morphogenesis (Jodoin *et al.,* 2015; Ikawa & Sugimura, 2018). However, how actin turnover and microtubules are coordinated to promote apical actomyosin contractility remains unclear and requires further investigation.

Relevance of our findings and similarities to other polarized cells

Our findings in the follicular epithelium indicate that CAP-mediated actin turnover is essential for the proper anchoring of microtubule minus-ends at cortical sites. This anchoring is vital for establishing and maintaining non-centrosomal microtubule arrays, crucial for cell polarity and intracellular transport.

Our current study enhances the understanding of CAP-mediated actin turnover in non-centrosomal microtubule organization. One of the earliest phenotypes associated with CAP loss-of-function was a defect in *Drosophila* oocyte polarity (Baum *et al.,* 2000). In *Drosophila*, oocyte polarity is established by non-centrosomal microtubule arrays, that transport polarity-determining mRNAs to the anterior and posterior ends (St Johnston, 2023). In CAP mutant oocytes, these mRNAs are mislocalized, suggesting a defect in microtubule polarity. However, until now, the mechanism by which CAP contributes to microtubule-related phenotypes has been incompletely understood.

Similarly to follicle cells, also in oocytes transition from centrosomally nucleated to non-centrosomal microtubule arrays, the establishment of which is critical for oocyte polarity. In CAP mutant oocytes, excessive actin accumulates at the anterior end (Baum et al 2000). Intriguingly, this is the cortical site where non-centrosomal microtubule arrays are normally anchored via Shot and Patronin (Nashchekin *et al.,* 2016). Our results show that in follicle cells CAP-mediated actin turnover is critical for proper localization of Shot and Patronin. Whether CAP-mediated actin turnover is also required for cortical localization of Shot and Patronin in oocytes remains to be investigated. However, it is tempting to speculated that establishment of non-centrosomal microtubule arrays in oocyte needs to be accompanied by CAP-mediated rapid actin filament turnover.

CAP is well-established for promoting actin dynamics in heart and skeletal muscle cells, as well as in neuronal cells. Its loss results in actin accumulation, which impairs muscle development and contraction, and affects neuronal growth cones and dendritic spines (Field *et al.,* 2015; Heinze *et al.,* 2022; Kepser *et al.,* 2019; Kumar *et al.,* 2016; Peche *et al.,* 2013; Schneider *et al.,* 2021). Furthermore, the loss of CAP activity, along with excessive actin accumulation, has been linked to diseases such as nemaline myopathy and Alzheimer's disease (Gurunathan *et al.,* 2022; Pelucchi *et al.,* 2020). Our study shows that disruption of actin turnover and excessive accumulation of actin in CAP mutant follicle cells can disrupt microtubule organization and microtubule-based transport. Interestingly, in both muscle and

neuronal cells, microtubules are organized in a non-centrosomal manner (Bugnard *et al.,* 2005; Kapitein & Hoogenraad, 2015; Stiess *et al.,* 2010; Tassin *et al.,* 1985). However, the impact of actin accumulation on microtubules has not been extensively studied in these cell types, except in neuronal cells, where persistent cofilin-actin rods disrupt microtubules and impair microtubule-based trafficking (Cichon *et al.,* 2012; Medina *et al.,* 2008; Minamide *et al.,* 2000). Therefore, future studies are essential to determine whether CAP plays a similar role in regulating non-centrosomal microtubule arrays in other cellular contexts, further elucidating the intricate interplay between actin dynamics and microtubule organization in cells.

MATERIALS AND METHODS

Drosophila stocks

All the fly lines used in this study were obtained from Bloomington *Drosophila* Stock Center or shared as gifts from other research groups. *Drosophila* fly line Capt^{E539}, an allele of Act up or Capulet (described as CAP) encoding a nonsense mutation at tryptophan 141 (described in Benlali *et al.,* 2000, BDSC 5944) was used to generate CAP (Cyclase-associated protein) chimeric loss-of-function mutant clones. Mitotic mutant clones in the follicular epithelium were generated using FLP/FRT systems, hsFLP; TubGal80, FRT40A; TubP-Gal4, UAS-mCD8-GFP/ TM6B, Tb (MARCM 40, Gifted by Pernille Rørth) and hsflp; UbimRFPnls FRT40/cyo. CAP loss of function clones were rescued with UAS fly lines encoding for wildtype CAP and specific point mutations that abolished different biochemical activities of CAP. These UAS-CAP fly lines were generated in-house for this project (see Table 1). UAS-Chic, encoding for profilin was kind gift from Lynn Cooley. Endosomal trafficking in mutant clones was analysed by combining Rab11-EYFP (BDSC 62549) with Capt^{E593}. Localization of F-actin crosslinker α-actinin is visualized by combining Capt^{E593} with α-actinin-GFP (BDSC 602268). Localization of AIP1 was analyzed using AIP1- protein trap AIP1GFP (BDSC 50824). Combining Capt^{E593} and Ubi-p63E.Patronin GFP (BDSC 55129) were used to analyze the localization of microtubule minus-end binding protein patronin. All fly stocks were maintained at 18°C and crossed at 25°C in a standard food medium.

Generation of CAP domain mutants

Site-directed mutagenesis of selected amino acids to alanins (Table 1) was performed within the longer cDNA of CAPT-RB (RH0878) isoform (Table S1). The parts corresponding to CAPT-RA isoform from the mutated CAPT-RB (RH0878, FlyBase ID FBtr0100023) isoform were cloned into the pUAST-attB vector. PCR was amplified using DNA polymerase enzyme KAPA HiFi (ReadyMix x 2, #KK2601, KAPA Biosystems; 30-60 sec/kb) following the recommended protocol. Resulting plasmids were verified with sequencing. The transgenic flies were generated by

BestGene inc. *Drosophila* embryo injection services.

Generation of mutant clones

For the generation of mutant clones, 2–4-day-old female flies were transferred to empty food vials and given two heat shocks at 37°C for one hour with a 5-hour interval between each heat shock. After the two heat shocks, flies were kept in dry yeast and allowed to recover for 3 days at 25°C. For rescue experiments clones were generated up to 9 days prior dissections, in order to ensure the disappearance of endogenous CAP. After the recovery ovaries were dissected from these flies. The flies were transferred to a fresh food medium containing wet yeast overnight before the dissection.

Generation of Capt antibody

Ig fractions of Capt-immunized rabbit serum (Pineda Antibody Service) were purified by affinity chromatography from resin-antigen-coated beads. 500 µL of fraction was eluted from the beads with 5 mL 0.1 M glycine-HCl (pH 2.8). The specificity of the collected fractions was determined with the western blot of fly lysates of wild type (W1118) and capt^{E593} lysates. Additionally, immunostaining with purified anti-Capt antibody showed decreased intensity in Capt^{E593} clones relative to the control cells.

Dissection and Immunostaining

Ovaries were dissected in ice-cold PBS and fixed in 4% paraformaldehyde for 20 min. For acetylated αtubulin staining, the ovaries were dissected in room temperature PBS and fixed in 8% paraformaldehyde for 10 min. For Dynein heavy chain (Dynein-HC) and Shot staining, ovaries were dissected in cold PBS and fixed for10 min using cytoskeleton buffer without glutaraldehyde, with 10% sucrose, 8% paraformaldehyde and 0.05% Triton-X, thereafter ovaries were washed once with PBS and quenched with sodium borohydride ($NaBH_4$) for 10 min. The egg chambers are permeabilized in 0.1% BSA in 0.5% PBST and blocked with blocking buffer 2.5% BSA in 0.5% PBST. The primary and secondary antibodies were diluted in the blocking buffer, and the samples were incubated overnight at 4°C and for 2 hours at room temperature respectively.

Primary antibodies used were rabbit anti-Capt (1:400, generated in the lab), mouse anti-acetylated αtubulin (1:100, Santa Cruz biotechnology 6-11B-1), rabbit anti-αTubulin (1:50, Abcam Ab18251), mouse anti-βTubulin (1:50, DSHB E7), mouse anti-Shot (1:25, DSHB mABRod1), mouse anti-Cnx99A (1:25, DSHB Cnx99A 6-2-1), mouse anti-Golgin84 (1:25, DSHB Golgin84 12-1), mouse anti-DyneinHC (1:25, DSHB 2C11-2), rabbit anti-Cad99C (1:2000, gift from Christian Dahmann lab; Schlichting *et al.,* 2005), mouse anti-profilin (1:25, DSHB chi 1J), Twinfilin (gift from Tapio Heino; Wahlström *et al.,* 2001), mouse anti-Ena (1:25, DSHB 5G2), rat anti-Tropomyosin (1:25, DSHB BB5/37.1) and rabbit anti-Phospho-Myosin II (1:300, Cell Signalling Technologies). Secondary antibodies, donkey anti-mouse Alexa 647 and goat anti-rabbit Alexa 647 (from Thermofisher Scientific),

were used in a 1:300 dilution. 1μg/mL DAPI and Phalloidin (1:300, from Thermofisher Scientific) for staining the nucleus and F-actin, respectively.

Transmission electron microscopy

Ovaries were dissected in cold 0.1M phosphate buffer (pH 7.4) and fixed in 2.5% glutaraldehyde (EM-grade) and 2% paraformaldehyde in 0.1M phosphate buffer for 1 hour at room temperature and then continued at 4°C overnight. Egg chambers were then washed with cold phosphate buffer on ice and post-fixed with 1% osmium tetroxide in phosphate buffer for 1 hour on ice, later washed with dH2O and stained with 1% uranyl acetate in dH2O at 4°C in the dark for 1 hour. After post-fixation and em block staining, egg chambers were dehydrated through a gradient of ethanol, rinsed with propylene oxide and gradually embedded into TAAB Hard epoxy resin. 60-nm thin sections were cut using an ultramicrotome (Leica EM Ultracut UC7, Leica Mikrosysteme GmbH, Austria), collected on Pioloform-coated single slot Copper grids and poststained with uranyl acetate and lead citrate. TEM imaging was done using JEM-1400 (JEOL Ltd, Japan) equipped with an Orius SC 1000B bottom-mounted CCD-camera (Gatan Inc., USA) at accelerating voltage of 80 kV and magnifications ranging from 8000 × to 100,000 ×.

Confocal microscopy and image analysis

Confocal images were acquired using Leica SP8 Upright microscope using 20x and 63x oil immersion objectives. Image processing and analysis were conducted using ImageJ software. Fluorescence intensity in the apical region was calculated as the average value of all pixels within the ROI spanning the apical subcellular region. Data for the Line scan intensity plots for CAP^{E593} were acquired from line ROIs parallel to the apicobasal axis from the cross-section of the follicle cells. The distance is adjusted such that 0 in the x-axis of the graphs corresponds to 25% of the total F-actin intensity. The signal intensities are normalised to the maximum of the control sample, and maximum-minimum normalisation was performed for the mean value for plotting. For quantifying the nuclear position, the distance between the center of the nucleus to the basal surface is measured as a proxy to show the shift in the position of the nucleus.

Statistical analysis

The outcomes were derived from a minimum of two autonomous experiments, employing multiple egg chambers for analysis with reproducibility. Before performing the statistical analysis normality of the data set was analyzed using the W test or the Shapiro-Wilk test. For the normally distributed data sets, parametric tests, paired t-tests were used and for non-parametric data sets, Mann-Whitney tests were used to assess the significance of differences between the control and the CAP^{E593}. For data with more than two groups Kruskal-Wallis H test or one-way ANOVA was conducted. The analysis was performed using OriginPro 2025 software.

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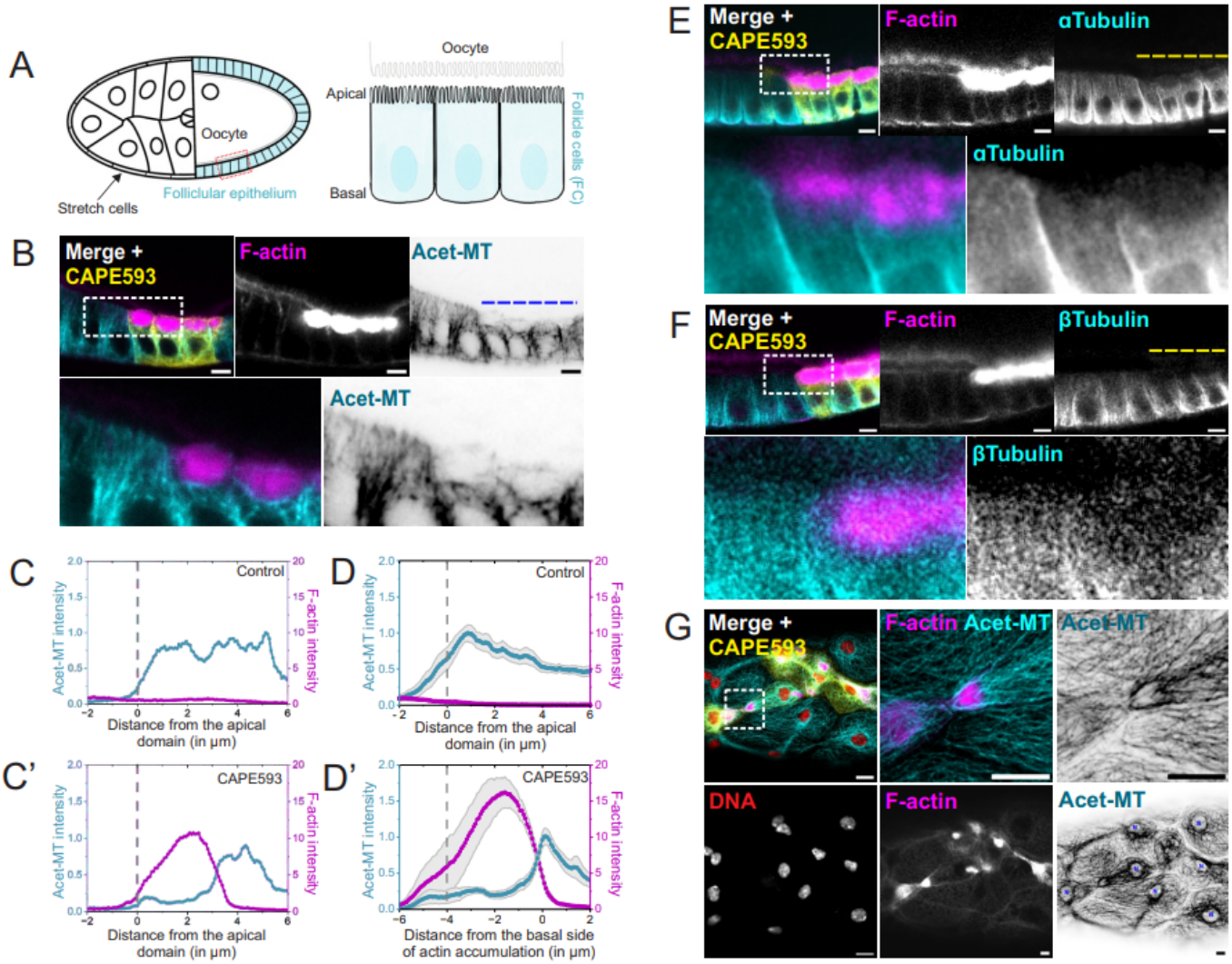

Figure 1. CAP loss of function results in F-actin accumulation and misorganized microtubules. (A) Illustration of stage 10 Drosophila melanogaster egg chamber highlighting the apicobasal polarization of the follicular epithelium. The black arrow points towards the squamous cells called stretch cells. **(B) Apical actin accumulation excludes acetylated microtubules in CAP mutant follicle cells.** A mosaic of follicular epithelium is shown, with CAP mutant cells highlighted by mCD8GFP (GFP positive, pseudo-colored yellow, dashed line). The merged image illustrates the accumulation of filamentous actin at the apical region, as indicated by Phalloidin staining (pseudo-colored magenta). In contrast, acetylated microtubules (pseudo-coloured cyan) are absent from this region. Scale bar = 10 µm. Zoomed-in inset (dotted box) highlights the site of actin accumulation (magenta) devoid of acetylated microtubules. **(C) Line scan intensity profile** of the F-actin (magenta) and acetylated microtubules (cyan) for a single representative control (C) and CAP mutant follicle cell (C'). The dotted line marks the apical boundary of the follicle cells. **(D) Mean line scan intensity profile** of F-actin (magenta) and acetylated microtubules (cyan) is presented for control (D) and CAP mutant follicle cells (D'). In (D'), the x-axis represents the distance from the basal side of the accumulated F-actin to normalise for the variation in the space occupied by the accumulated actin in the cells. The dotted

line marks the apical boundary of the follicle cells. Data are presented as mean ± SEM; n = 5 cells, N = 2 egg chambers. **(E, F) Apical actin accumulation excludes microtubule α- and β-tubulin subunits in CAP mutant follicle cells** (GFP positive, pseudo-colored yellow, dashed lines). Zoomed-in inset (dotted box) shows apical region CAP mutant cells with actin accumulation (Phalloidin, pseudo-colored magenta) and the site of apical actin accumulation is devoid of (D) α-Tubulin (cyan), and (E) β-Tubulin (cyan). Scale bar = 10 μm. **(G) Actin accumulation excludes acetylated microtubules in CAP mutant stretch cells.** Stretch cells containing CAP mutant cells are marked by mCD8GFP (GFP positive, pseudo-colored yellow). F-actin (phalloidin-stained, pseudo-colored magenta) accumulates at certain regions of the stretch cell cytoplasm. Zoomed-in inset (dotted box) highlights a site of filamentous actin accumulation (detected with Phalloidin, magenta) in the stretch cell is devoid of stable acetylated microtubules (Acet-MT, cyan). Additionally, acetylated microtubules are absent from the vicinity of the stretch cell nuclei, which are stained with DAPI (pseudo-colored red in the merged image and labelled "N" in the inverted greyscale LUT image). Scale bar = 10 μm.

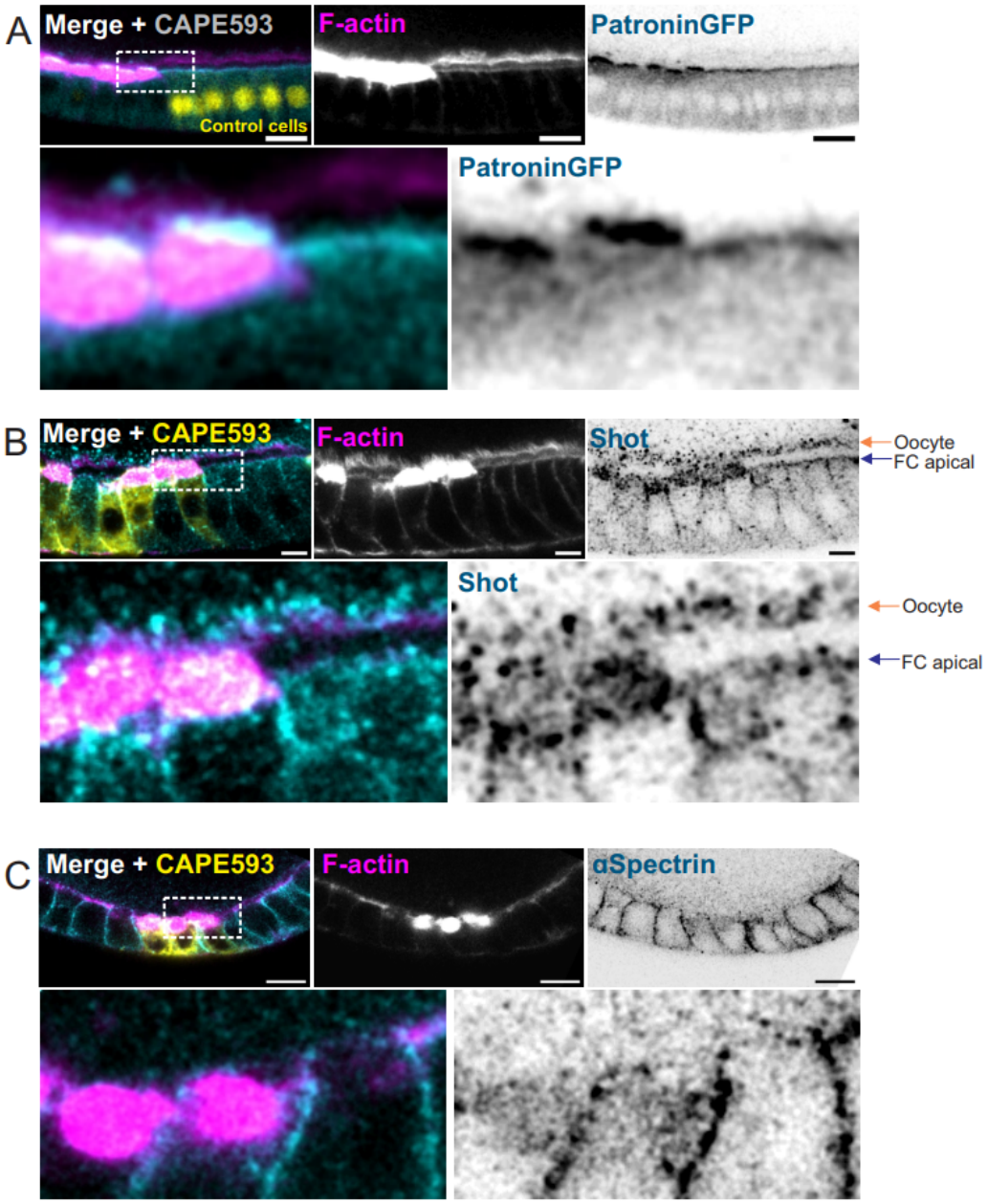

Figure 2. Localisation of microtubule minus-end anchoring proteins Patronin and actin-microtubule crosslinking protein Shot is impaired in CAP mutant cells. (A) Apical localisation of microtubule minus-end anchoring protein Patronin. A mosaic of follicular epithelium is shown with CAP mutant cells marked by the absence of nuclear RFP (nlsRFP (pseudocoloured yellow) negative). The merged image illustrates the accumulation of filamentous actin at the apical region, as indicated by Phalloidin staining (pseudo-colored magenta). Zoomed-in inset (dotted box), shows that the microtubule minus-end binding protein Patronin (Patronin-GFP, pseudo-colored cyan) is localized apically in both control cells (nlsRFP positive, pseudo-colored yellow) and CAP mutant cells (nlsRFP negative). However, the intensity of Patronin-GFP is higher in CAP mutant cells, and its localisation appears more diffuse. Scale bar = 10 μm. **(B) Impaired apical localisation of actin-microtubule crosslinking protein Shot.** Mosaic follicular

epithelium with CAP mutant cells (GFP positive) staining F-actin (phalloidin) and antibody against Shot. The zoomed-in inset shows the disrupted apical localisation of actin-microtubule crosslinker Shot (pseudo-colored, cyan) at the site of actin accumulation. Shot is localised basal to the accumulated actin. The orange arrow highlights the Shot localisation on the oocyte cortex, and the blue arrow highlights the localisation of Shot on the apical surface of the follicle cells. Scale bar = 10 μm. **(C) Disrupted apical localisation of α-spectrin.** Mosaic follicular epithelium with CAP mutant cells (GFP positive) showing F-actin accumulation (phalloidin staining) and immunostaining against α-spectrin. Zoomed-in inset highlights the disrupted localisation of α-spectrin at the site of actin accumulation. Scale bar = 10 μm.

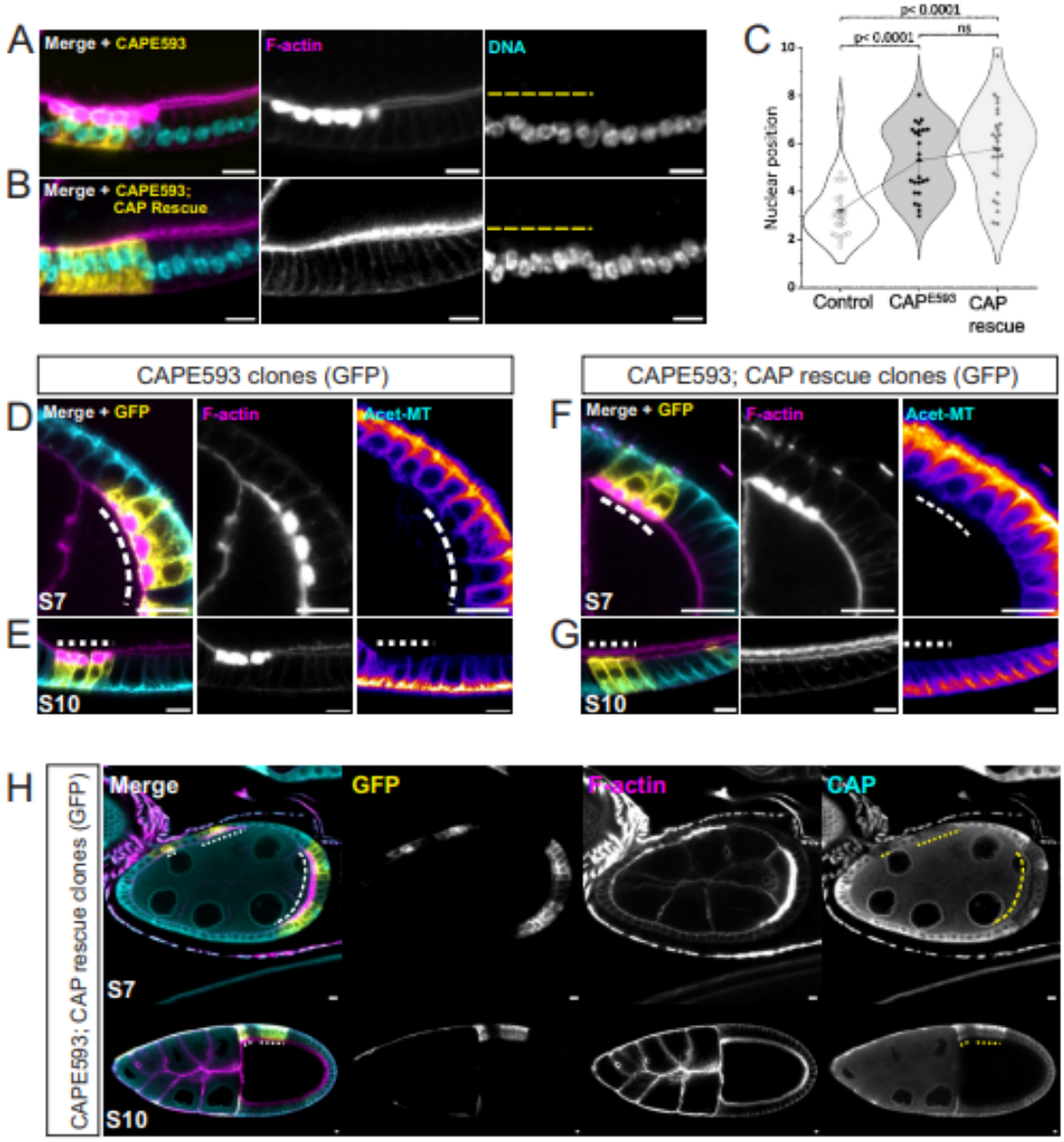

Figure 3. Apical actin accumulation during early oogenesis causes mispositioning of follicle cell nuclei. (A) CAP mutant cell nuclei are mispositioned apically. Mosaic follicular epithelium with CAP mutant cells marked with GFP (GFP positive, pseudo-colored yellow, dashed line) and filamentous actin (stained with phalloidin, pseudo-colored magenta). In CAP mutant cells, nuclei (DNA stained with DAPI, pseudo-colored cyan) are positioned apically, while neighboring control cells (GFP negative) exhibit more basal nuclear positioning. Scale bar = 10 µm. **(B) Restoring CAP expression in CAP mutant cells rescues actin accumulation, but not the nuclear positioning phenotype.** Mosaic follicular epithelium with CAP mutant cells rescued by wild-type CAP expression (CAP rescue, GFP positive, pseudo-colored yellow, dashed line). While apical actin accumulation (filamentous actin stained with phalloidin, pseudo-colored magenta) is restored, the nuclei (DNA, cyan) remain mislocalized to the apical domain. Scale bar = 10 µm. **(C) Quantification of nuclear position in control, CAP mutant, and rescued cells.** A quantification graph is presented showing the nuclear position defined by the distance from the

nucleus's centre to the cell's basal side. The connecting line passes through the mean value of each group, and the whiskers represent the standard deviation. Kruskal–Wallis ANOVA followed by Dunn's post hoc test was used to assess the statistical significance of the different groups. Control = 25 cells, CAPE593 = 25 cells, and CAP rescue = 26 cells. N = 6 egg chambers. **(D, E) CAP mutant cells display accumulated actin and microtubule disorganisation in both stage 7 and stage 10.** A cross-section of (D) stage 7 and (E) stage 10 mosaic follicular epithelium is shown, with CAP mutant cells highlighted by cd8GFP (GFP positive, dashed line). In both stages, CAP mutant cells display apical actin accumulation (filamentous actin stained with phalloidin, pseudo-colored magenta) and absence of acetylated microtubules (Acet-MT, pseudo-colored cyan). Scale bar = 10 µm. **(F) Actin accumulation and microtubule organization phenotypes are not rescued during early oogenesis.** A cross-section of stage 7 (s7) follicular epithelium, with CAP rescue clones highlighted by cd8GFP (GFP positive, pseudo-colored yellow, dashed line). The merged image demonstrates that apical actin accumulation is not rescued (filamentous actin stained with phalloidin, pseudo-colored magenta) nor is the organization of stable acetylated microtubules (Acet-MT, pseudo-colored cyan) in stage 7. Scale bar = 10 µm. **(G) Actin accumulation and microtubule organization phenotypes are successfully rescued in later stages of oogenesis.** A cross-section of stage 10 (s10) follicular epithelium is shown, with CAP wildtype rescue clones highlighted by cd8GFP (GFP positive, pseudo-colored yellow, dashed line). Both apical actin accumulation (filamentous actin stained with phalloidin, pseudo-colored magenta) and stable acetylated microtubules (Acet-MT, pseudo-colored cyan) are successfully restored. Scale bar = 10 µm. **(H) Lack of CAP rescue construct expression during early oogenesis**. Cross-sections of stage 7 (s7) and stage 10 (s10) egg chambers, with CAP rescue clones highlighted by cd8GFP (GFP positive, dashed line). The CAP rescue transgene is expressed (as indicated by immunostaining, pseudo-colored cyan) and rescues actin accumulation in stage 10. In stage 7, the CAP rescue construct is not expressed and thus fails to rescue the actin accumulation phenotype. Scale bar = 10 µm.

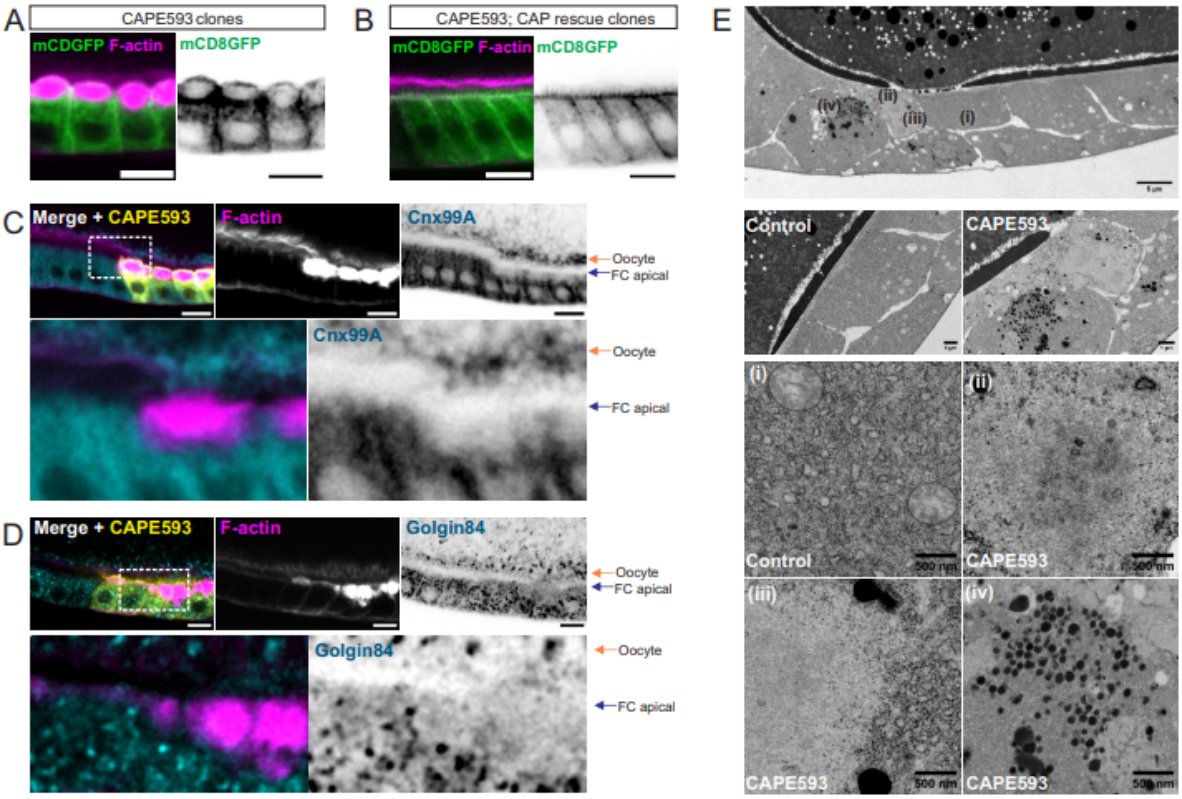

Figure 4. Membrane-bound organelles are excluded from site of actin accumulation (A) Absence of membrane-bound cd8GFP at site of actin accumulation in CAP mutant cells. A mosaic of follicular epithelium with CAP mutant cells marked with mCD8GFP (GFP positive, pseudo-colored yellow, dashed line) and filamentous actin (stained with phalloidin, pseudo-colored magenta). In CAP mutant cells, the membrane bound mCD8GFP clonal marker (green) is absent in the region of actin accumulation (magenta). Scale bar = 10 µm. **(B) mCD8GFP localization is restored upon CAP rescue. Mosaic follicular epithelium expressing wildtype CAP in CAP mutant cells.** CAP rescue (mCD8GFP positive cells) restores the actin accumulation phenotype (F-actin, magenta) and re-establishes mCD8GFP (green) localization. Scale bar = 10 µm. **(C) The site of actin accumulation lacks ER markers.** Mosaic follicular epithelium with CAP mutant cells (GFP positive, pseudo-colored yellow) stained for F-actin (phalloidin) and antibody against ER marker Calnexin99A (Cnx99A, pseudocoloured, cyan). Zoomed-in inset shows the apical region of the cell with a lack of ER markers at the site of F-actin accumulation. The orange arrow highlights the Cnx99A localisation on the oocyte cortex, and the blue arrow highlights the localisation of Cnx99A on the apical surface of the follicle cells. Scale bar = 10 µm. **(D) The Golgi marker is lacking at the site of actin accumulation.** Mosaic follicular epithelium with CAP mutant cells (GFP positive, pseudo-colored yellow) stained for F-actin (phalloidin) and antibody against Golgi marker Golgin84 (pseudocoloured, cyan). Zoomed-in inset shows the apical region of the cell with F-actin accumulation, lacking the Golgi marker Golgin84. The orange arrow highlights the Golgin84 localisation on the oocyte cortex, and the blue arrow highlights the localisation of Golgin84 on the apical surface of the follicle cells. Scale bar = 10 µm. **(E) Transmission electron microscopy (TEM) of CAP mutant cells.** Transmission electron microscopy (TEM) image of follicular epithelium with CAP mutant clones and control cells. The site of actin accumulation appears less dense than other regions of the cell and neighboring control cells and lacks organelles such as mitochondria, ER, and ribosomes. The

numbered regions correspond to specified magnified insets. **(i)** Magnified region near the apical domain of the control cell, showing the presence of membrane-bound organelles like mitochondria. **(ii)** Magnified image of the region near the apical domain in CAP mutant cells, showing the lack of cytoplasmic components. **(iii)** Magnified image of the boundary of the site of actin accumulation, highlighting the difference in cytoplasmic components inside and outside of the accumulated actin. **(iv)** Magnified image showing the presence of electron-dense vesicular structures near the site of actin accumulation in CAP mutant cells.

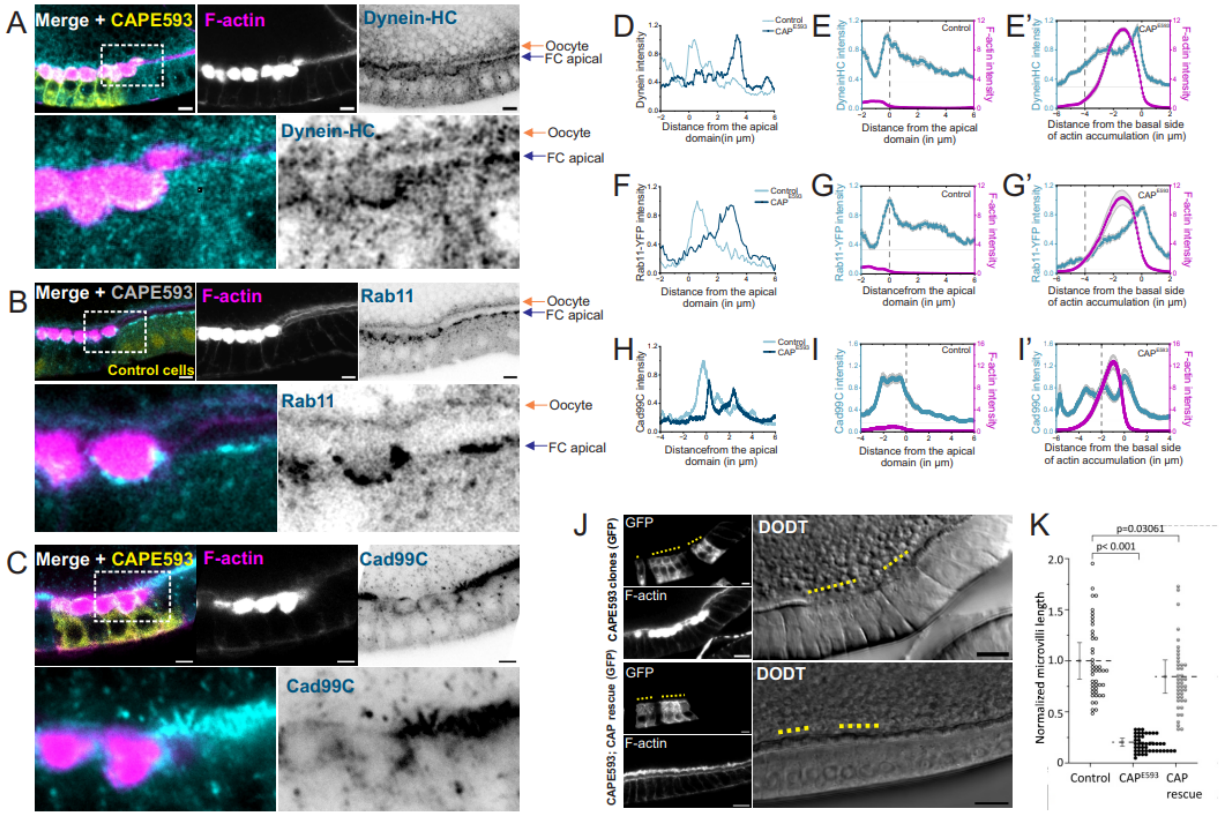

Figure 5. Apical actin accumulation halts Dynein and Rab11-mediated trafficking, leading to the retention of apical cargo Cad99c required for microvilli biogenesis (A) Dynein accumulates basally to the actin accumulation. Mosaic follicular epithelium with CAP mutant cells (GFP positive, pseudocoloured yellow) stained for F-actin (magenta) with phalloidin and immunostained against motor protein dynein heavy chain (Dynein-HC, pseudocoloured cyan). Zoomed-in inset (dotted box) shows the accumulation of Dynein beneath the apically accumulated F-actin. The orange arrow highlights the Dynein-HC localisation on the oocyte cortex, and the blue arrow highlights the Dynein-HC localisation on the apical surface of the follicle cells. Scale bar = 10 µm. **(B) Rab11-positive endosomes localise beneath apical actin in CAP mutant cells**. Mosaic follicular epithelium with CAP mutant cells marked by the absence of nuclear RFP (nlsRFP negative, pseudo-colored yellow), expressing YFP-tagged Rab11(pseudo colored cyan), stained with phalloidin against F-actin. Zoomed-in inset (dotted box) shows that the apically accumulated F-actin halts the apical trafficking of Rab11 endosomes. Scale bar = 10 µm. **(C) Microvilli-specific atypical Cad99C localises beneath accumulated actin in CAP mutant cells**. Mosaic follicular epithelium with CAP mutant cells (GFP, pseudo-colored yellow, dashed line) stained for F-actin (phalloidin, magenta) and immunostaining against microvilli-specific atypical Cadherin, Cad99C (cyan). Zoomed-in inset (dotted box) highlights the retention of Cad99C (cyan), below the accumulated F-actin (magenta) and failure to reach the apical side of the follicle cells. Scale bar = 10 µm. **(D) Line scan intensity profile of dynein in control and CAP mutant cells.** The intensity profile of Dynein heavy chain is plotted for representative control (light cyan) and CAP mutant (dark cyan) cells, revealing a shift in Dynein localisation at the apical region of CAP mutant cells.**(E) Comparative line scan of filamentous actin and dynein.** Mean line scan intensity profiles of F-actin (magenta) and Dynein (cyan) are presented for (E) control and (E') CAP mutant follicle cells. In (E'), the x-axis represents the distance from the basal side of the accumulated F-actin to normalise for the variation in the space occupied by the accumulated

actin in the cells. The dotted line marks the apical boundary of the follicle cells, just below the actin-rich microvilli. Data are presented as mean ± SEM; n = 11 cells, N = 4 egg chambers. **(F) Line scan intensity profile of Rab11 in control and CAP mutant cells**. The intensity profile of Rab11-YFP is plotted for representative control (light cyan) and CAP mutant (dark cyan) cells, revealing a shift in Rab11 localisation at the apical region of CAP mutant cells. **(G) Comparative line scan of filamentous actin and Rab11.** Combined line scan intensity profiles of F-actin (magenta) and Rab11-YFP (cyan) for (G) control and (G') CAP mutant follicle cells. In (G'), the x-axis represents the distance from the basal side of the accumulated F-actin to normalise for the variation in the space occupied by the accumulated actin in the mutant cells. The dotted line marks the apical boundary of the follicle cells, just below the microvilli. Data are presented as mean ± SEM; n = 20 cells, N = 6 egg chambers. **(H) Line scan intensity profile of Cad99C in control and CAP mutant cells**. The intensity profile of Cad99C is plotted for representative control (light cyan) and CAP mutant (dark cyan) cells, revealing a shift in Rab11 localisation at the apical region of CAP mutant cells**. (I) Comparative line scan of filamentous actin and Cad99C.** Mean line scan intensity profiles of F-actin (magenta) and Cad99C (cyan) for (I) control and (I') CAP mutant follicle cells. In (I'), the x-axis represents the distance from the basal side of the accumulated F-actin to normalise for the variation in the space occupied by the accumulated actin in the mutant. The dotted line marks the apical-most boundary of the follicle cells, just below the microvilli (which in control cells are Cad99C positive). Data are presented as mean ± SEM; n = 15 cells, N = 6 egg chambers. **(J) Loss of apical microvilli in CAP mutant cells.** Mosaic follicular epithelium with CAP mutant cells (GFP positive, dashed line). The gradient contrast (DODT) image shows the loss of apical microvilli in CAP mutant cells (dashed line). CAP mutant cells rescued with wild-type CAP expression (CAP rescue, GFP positive, dashed line) show the rescue of F-actin accumulation and apical microvilli. Scale bar = 10 µm. **(K) Quantification of microvilli length relative to control.** Quantification of microvilli length normalised with the control. The bar shows the ± 0.5 SD, and the dotted line represents the mean value. One-way ANOVA with post-hoc test was used to determine the statistical significance between different groups. Control = 45 cells, CAPE593 = 45 cells; CAP rescue = 43 cells. N = 9, 11, 7 egg chambers.

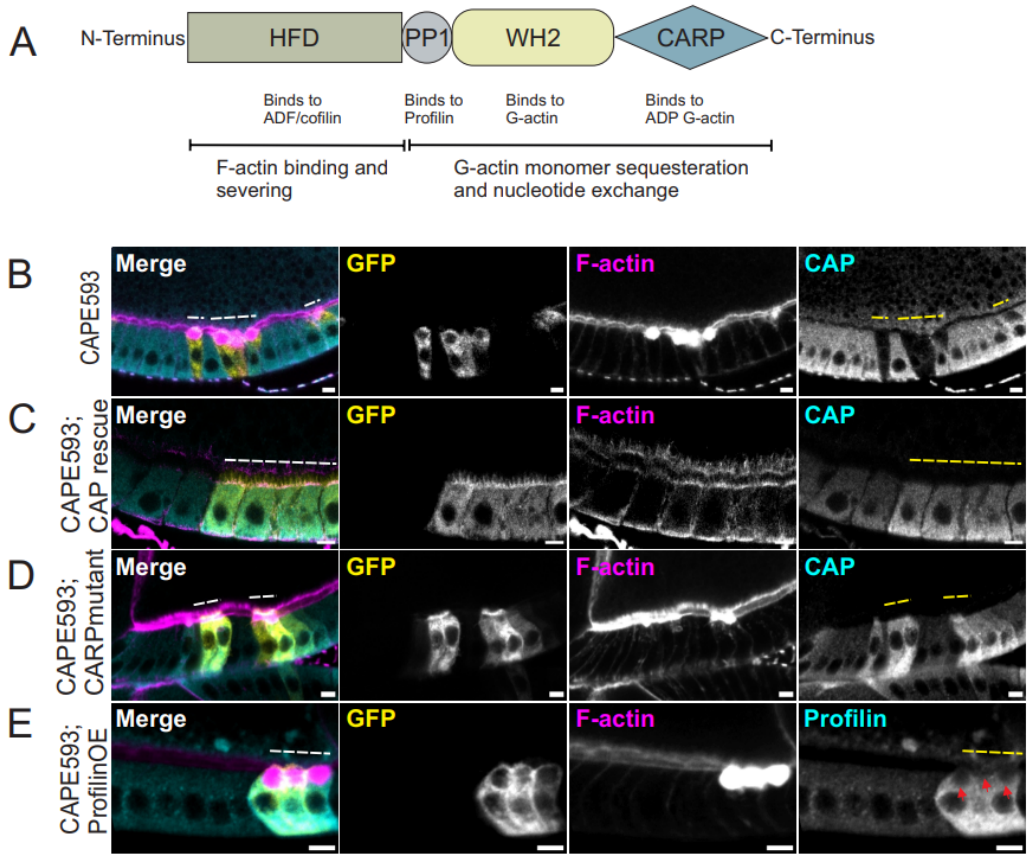

Figure 6. Nucleotide exchange activity of CAP is critical for apical actin turnover. (A) Schematic diagram of protein domain organization of CAP. CAP is a multidomain protein, with each domain exhibiting distinct biochemical activities that contribute to actin turnover. The N-terminal Helical Folded Domain (HDF) enhances depolymerization of cofilin-decorated actin filaments by dissociating cofilin-actin monomers (Bertling *et al.,* 2004; Chaudhry *et al.,* 2013; Jansen *et al.,* 2014; Kotila *et al.,* 2019; Quintero-Monzon *et al.,* 2009; Shekhar *et al.,* 2019). The Polyproline (PP) domain binds profilin. The Wiskott-Aldrich Syndrome Protein Homology 2 (WH2) domain interacts with both ADP-actin and ATP-actin monomers (Bertling *et al.,* 2007; Freeman *et al.,* 1995; Lila & Drubin, 1997; Mattila *et al.,* 2004). The CARP domain binds ADP-actin monomers and facilitates nucleotide exchange from ADP to ATP, thereby recharging actin monomers for polymerization (Moriyama & Yahara, 2002; Bertling *et al.,* 2004; Kotila *et al.,* 2018). **(B) CAP mutant cells show apical actin accumulation**. Mosaic follicular epithelium with CAP mutant cells (GFP, pseudo-colored yellow, dashed line) exhibiting significant apical actin accumulation (Phalloidin, pseudo-colored magenta) alongside a loss of CAP expression (immunostaining, pseudo-colored cyan). Scale bar = 10 µm. **(C) CAP rescue restores apical actin accumulation.** Mosaic follicular epithelium with CAP mutant cells rescued with wildtype CAP (GFP positive, pseudo-colored yellow, dashed line) demonstrating restored apical actin accumulation (Phalloidin, pseudo-colored magenta) and expression of the CAP rescue construct (immunostaining, pseudo-colored cyan). Scale bar = 10 µm. **(D) CARP domain mutant fails to rescue apical actin accumulation.** Mosaic follicular epithelium with CAP mutant cells rescued with a CARP domain mutant of CAP (GFP positive, pseudo-colored yellow, dashed line) showing failure to restore apical actin accumulation (Phalloidin, pseudo-colored magenta). Expression of the CAP CARP-domain rescue construct is depicted (immunostaining, pseudo-colored cyan). Scale bar = 10 µm. **(E) Profilin overexpression fails to rescue apical actin accumulation.** Mosaic follicular epithelium with CAP mutant cells overexpressing profilin (GFP positive, pseudo-colored yellow,

dashed line) demonstrating failure to restore apical actin accumulation (Phalloidin, pseudo-colored magenta). The expression of the profilin construct is shown (immunostaining, pseudo-colored cyan). The profilin expression is increased at the basal region of the cells. Red arrows denote the actin accumulation site lacking profilin localisation. Scale bar = 10 μm.

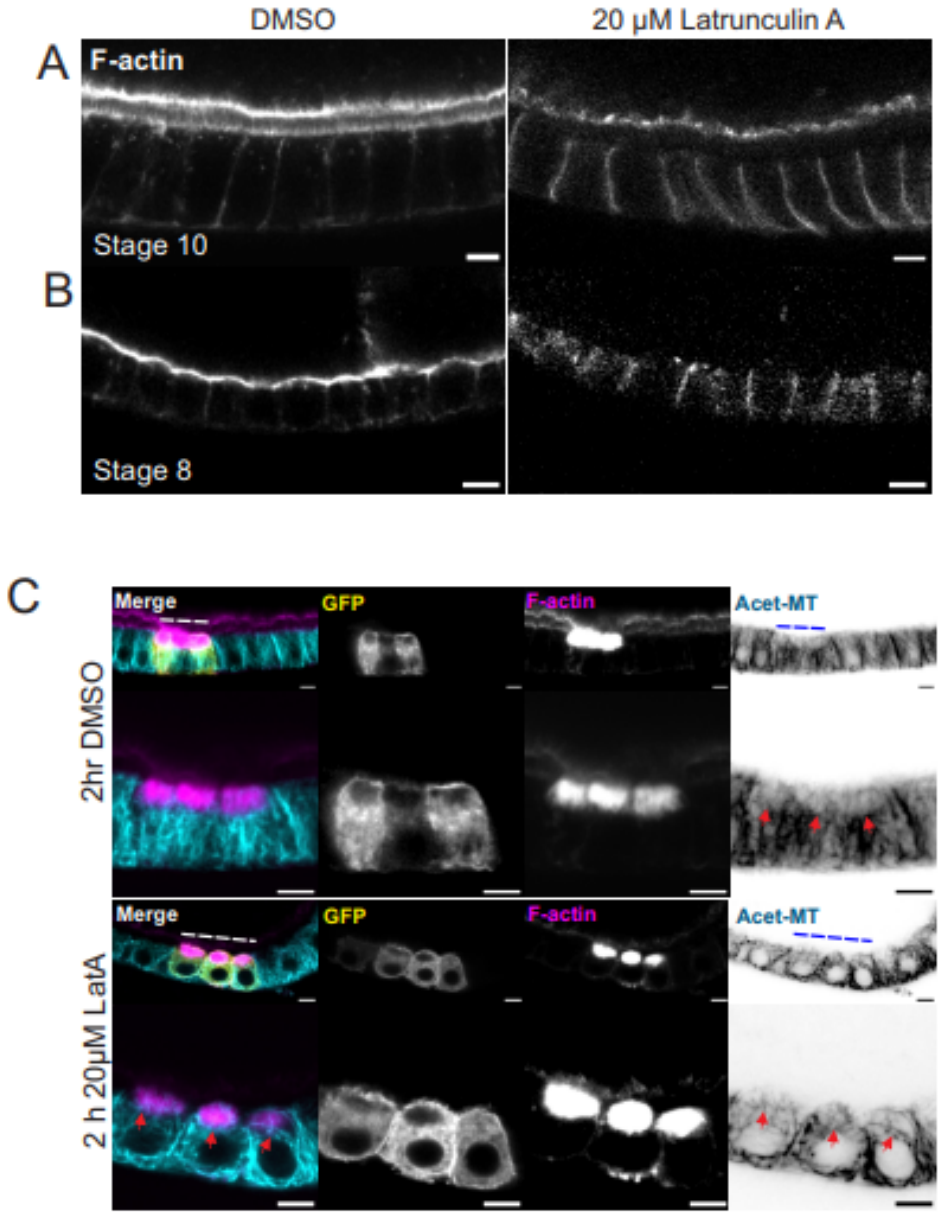

Figure 7. Loss of CAP disrupts fast turnover of apical actin, leading to persistent dense actin that prevents microtubule entry. (A, B) Latrunculin A treatment reveals filamentous actin structures that are under fast turnover. Treatment of egg chambers with DMSO (control) and 20 μM Latrunculin A for 30-minute treatment results in a decrease in filamentous actin structures under fast turnover. (A) In stage 10 follicular epithelium, filamentous actin is decreased in apical microvilli, apical actin cortex, and basal stress fibres, while junctional actin and the oocyte actin cortex appear to be less affected. (B) In stage 8 follicular epithelium, Latrunculin A induces depletion of both apical actin and basal actin in the follicle cells, while junctional actin remains unaffected. Scale bar = 10 μm. **(C) Latrunculin A treatment fails to resolve dense accumulated actin of CAP mutant cells but facilitates microtubule entry.** Mosaic follicular epithelium with CAP mutant cells marked with mCD8GFP (GFP positive, pseudo-colored yellow, dashed line), stained for filamentous actin (stained with phalloidin, pseudo-colored magenta) and for acetylated microtubules (Acet-MT, pseudo-colored cyan), treated with DMSO (control) and 20

μM Latrunculin A for 2 hours. In DMSO-treated egg chambers, the site of actin accumulation lacked the microtubules. Upon treatment with Latrunculin A, acetylated microtubules are seen at the site of accumulation and membrane-bound mCD8GFP is also localised at the site of actin accumulation, showing partial disassembly of the accumulated actin. Scale bar = 10 μm.

Table 1: CAP domain mutations

Domains	Mutation	Reference	Function
HFD (90) Interface1	F122A, Y123A	(Kotila *et al.,* 2019; Quintero-Monzon *et al.,* 2009)	Cofilin-actin interaction Pointed end depolymerization
PP	^{179}PPPPPPP^{190} → ^{179}AAAAAAA^{190}		Profilin binding
WH2 (101)	K225A K226A	(Mattila *et al.,* 2004)	ATP-actin binding
CARP (m6)	K315A N317A D322A	(Kotila *et al.,* 2018)	ADP-actin binding, nucleotide exchange

Table 2: *Drosophila* genotypes used

Figure	Panel	Genotype	Abbreviation	Source
1	B, E, F, G	w,hsFLP/w; TubGal80, FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-mCD8-GFP / +	CAP^{E593}	BDSC 5944
2	A	hsflp;UbimRFPnls,FRT40/CaptE593 FRT40A; ubi.patronin-GFP/+	CAP^{E593}; PatroninGFP	BDSC 5944 BDSC 55129
	B, C	yw,hsFLP/w;TubGal80,FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-mCD8-GFP / +	CAP^{E593}	BDSC 5944
3	A, D, E	w,hsFLP/w;TubGal80,FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-mCD8-GFP / +	CAP^{E593}	BDSC 5944
	B, F, G, H	yw,hsFLP/w;TubGal80,FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-Capt/ UAS-mCD8-GFP	CAP^{E593}; CAP Rescue	BDSC 5944 UAS-Capt, in this ms.
4	A, C, D, E	w,hsFLP/w; TubGal80, FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-mCD8-GFP / +	CAP^{E593}	BDSC 5944
	B	yw,hsFLP/w;TubGal80,FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-Capt/ UAS-mCD8-GFP	CAP^{E593}; CAP Rescue	UAS-Capt, in this ms
5	A, C, J	w,hsFLP/w; TubGal80, FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-mCD8-GFP / +	CAP^{E593}	BDSC 5944
	B	hsflp; UbimRFPnls,FRT40/Capt^{E593} FRT40A; Rab11EYFP/+	CAP^{E593}; Rab11YFP	BDSC 5944 BDSC 62549
	J	yw,hsFLP/w;TubGal80,FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-Capt/ UAS-mCD8-GFP	CAP^{E593}; CAP Rescue	BDSC 5944 UAS-Capt, in this ms
6	B	yw,hsFLP/w;TubGal80,FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-mCD8-GFP / +	CAP^{E593}	BDSC 5944

	C	yw,hsFLP/w;TubGal80,FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-Capt/ UAS-mCD8-GFP	CAP^{E593}; CAP Rescue	BDSC 5944 UAS-Capt, in this ms
	D	yw,hsFLP/w;TubGal80,FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-Captm6/ UAS-mCD8-GFP	CAP^{E593}; CARPmutant	BDSC 5944 UAS-Captm6, in this ms
	E	yw,hsFLP/w;TubGal80,FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-Chic/ UAS-mCD8-GFP	CAP^{E593}; ProfilinOE	BDSC 5944 Lynn Cooley
7	A,B	w1118	w1118	
	C	yw,hsFLP/w;TubGal80,FRT40A/Capt^{E593} FRT40A; TubP-GAL4, UAS-mCD8-GFP / +	CAP^{E593}	BDSC 5944