Shot and Patronin polarise microtubules to direct membrane traffic and biogenesis of microvilli in epithelia

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Summary

In epithelial tissues, polarisation of microtubules and actin microvilli occurs along the apical-basal axis of each cell, yet how these cytoskeletal polarisation events are coordinated remains unclear. Here we examine the hierarchy of events during cytoskeletal polarisation in *Drosophila* and human epithelia. Core apical-basal polarity determinants polarise the Spectrin cytoskeleton to recruit the microtubule-binding proteins Patronin (CAMSAP1/2/3 in humans) and Shortstop (Shot; MACF1/BPAG1 in humans) to the apical membrane domain. Patronin and Shot then act to polarise microtubules along the apical-basal axis to enable apical transport of Rab11 endosomes by the Nuf-Dynein microtubule motor complex. Finally, Rab11 endosomes are transferred to the MyoV actin motor to deliver the key microvillar determinant Cadherin99C to the apical membrane to organise the biogenesis of actin microvilli.
**Introduction**

Cells in epithelial tissues are polarised and display distinct apical and basolateral membrane domains (Martin-Belmonte and Mostov, 2008; Rodriguez-Boulan and Macara, 2014; St Johnston and Ahringer, 2010; Tepass, 2012). How this fundamental apical-basal polarity is elaborated to direct the polarisation of all other features of epithelial cells remains a major unsolved problem (Nance and Zallen, 2011). For example, many epithelial cells exhibit polarisation of the Spectrin and microtubule cytoskeletons along the apical-basal axis, as well as polarisation of the actin cytoskeleton to produce distinctive apical microvilli. While the molecular assembly of Spectrins, F-actin microvilli and acentrosomal microtubules have been intensely studied, how these cytoskeletal features become polarised remains unclear (Bartolini and Gundersen, 2006; Sauvanet et al., 2015; Suozzi et al., 2012; Thomas, 2001).

In the case of the Spectrin cytoskeleton, polarisation was first observed in *Drosophila* epithelial cells, where an apical beta-Heavy (\(\beta_H\))-Spectrin subunit and basolateral beta-Spectrin subunit segregate into complementary cortical domains (Lee et al., 1997; Thomas and Kiehart, 1994a). Both types of beta subunit can dimerise with alpha (\(\alpha\))-Spectrin to form a spring-like network that interacts with FERM domain proteins and transmembrane proteins such as Crumbs (reviewed in (Bennett and Healy, 2009). Spectrins have been shown to function in maintaining membrane tension and in regulating signalling through the Crumbs-Hippo pathway, but whether there is a role for Spectrins in controlling apical-basal polarity has proven elusive (Deng et al., 2015; Fletcher et al., 2015; Krieg et al., 2014; Medina et al., 2002; Thomas et al., 1998; Wong et al., 2015; Zarnescu and Thomas, 1999b). Recent work suggests that basolateral Spectrins act with Integrins to promote columnar cell shape in *Drosophila* follicle cells (Ng et al., 2016). The apical FERM domain proteins have been linked to organisation of the actin cytoskeleton and microvilli in both *Drosophila* and mammalian cells, but whether the Spectrin cytoskeleton is also involved in this process remains unclear (Claret et al., 2014; Gloerich et al., 2012; Hipfner et al., 2004; Karagiosis and Ready, 2004; Polesello et al., 2002; Roch et al., 2010; Speck et al., 2003; ten Klooster et al., 2009).

In the case of the microtubule cytoskeleton in epithelial cells, the centrosomal nucleation of the mitotic spindle evident during mitosis gives way to an acentrosomal nucleation of polarised microtubules at the apical and basal plasma membranes.
during interphase, a process first noticed by electron microscopy studies in *Drosophila* (Mogensen and Tucker, 1987; Mogensen et al., 1993; Mogensen et al., 1989). Acentrosomal nucleation of microtubules was later demonstrated to occur in many eukaryotic organisms, from yeast to human cells (Carazo-Salas and Nurse, 2006; Mahoney et al., 2006; Reilein et al., 2005; Schuh and Ellenberg, 2007; Stiess et al., 2010). Nevertheless, the molecular system responsible for polarising microtubules in epithelial cells is still to be identified. Consequently, it has been difficult to genetically test the functional role of polarised microtubules in epithelia. Instead, mutation of the microtubule minus-end directed motor protein Dynein, or its adaptors, has been used to demonstrate a requirement for polarised microtubules in apical mRNA transport and positioning of the nucleus in *Drosophila* epithelia (Bullock and Ish-Horowicz, 2001; Dix et al., 2013; Holt and Bullock, 2009; Horne-Badovinac and Bilder, 2008; Liu et al., 2013; Mosley-Bishop et al., 1999; Swan et al., 1999; Wilkie and Davis, 2001). There also appears to be a role for Dynein in trafficking E-cadherin during early polarity establishment and during tracheal morphogenesis (Harris and Peifer, 2005; Le Droguen et al., 2015). The overall apical-basal polarisation of epithelial cells is sometimes affected in *dynein* mutants, which resemble *crumbs* mutants that mostly polarise normally but occasionally lose polarity and become multilayered (Bullock and Ish-Horowicz, 2001; Fletcher et al., 2012a; Horne-Badovinac and Bilder, 2008; Wilkie and Davis, 2001). Accordingly it was proposed that Dynein trafficks mRNA for Stardust, a Crumbs binding partner (Horne-Badovinac and Bilder, 2008). These results raise the question of whether polarised microtubules are truly essential for polarised trafficking and localisation of membrane proteins, as has often been suggested based on observations of membrane trafficking in mammalian epithelial cells in culture (Mostov et al., 2000; Rodriguez-Boulan et al., 2005).

In the case of apical microvilli, the specific microvillar protocadherin PCDH15 was identified by human genetic studies of Usher syndrome, an inherited deaf-blindness disease caused by defects in stereocilia of the human ear cochlear cells and microvilli of the eye photoreceptor cells (Alagramam et al., 2001a; Alagramam et al., 2001b; Ben-Yosef et al., 2003). PCDH15 interacts with CDH23 to form tip-link filaments in stereocilia that are necessary for hearing (Elledge et al., 2010; Geng et al., 2013; Kazmierczak et al., 2007; Sollner et al., 2004). The *Drosophila* PCDH15 homologue is named Cadherin99C (Cad99C) and is necessary for normal biogenesis of microvilli, and is also sufficient to expand microvilli length when overexpressed (Chung and Andrew, 2014; D’Alterio et al., 2005; Schlichting et al., 2006). To perform
its function, Cad99C/PCDH15 interacts with the actin motor protein Crinkled/MyosinVIIA, which is encoded by the human MYO7A gene that is also mutated in Usher syndrome patients (Glowinski et al., 2014). Yet, how Cad99C/PCDH15 becomes localised to the apical domain of epithelial cells remains a mystery.

Here we show that polarised microtubules are essential to direct trafficking of Cad99C to apical microvilli in *Drosophila*. We identify the microtubule-binding proteins Patronin (CAMSAP 1/2/3 in humans) and Shortstop (Shot or MACF1/BPAG1 in humans) as acting in parallel at the apical domain of epithelial cells to polarise microtubules and delivery of Cad99C. We further show that polarisation of Patronin and Shot is dependent on the apical spectrin cytoskeleton, which in turn is dependent of determinants of cell polarity. Cad99C is transported apically inside Rab11 endosomes, which are linked to the Dynein microtubule motor protein via its adaptor protein nuclear fallout (Nuf). Once at the apical cortex, Rab11 endosomes are transferred to the MyoV actin motor complex to enable delivery of Cad99C to the apical plasma membrane. Our findings reveal a novel mechanism linking epithelial cell polarity with polarisation of the spectrin and microtubule cytoskeleton to direct apical membrane trafficking and biogenesis of microvilli.
Results

We began by examining the biogenesis of apical microvilli in the *Drosophila* ovarian follicle cell epithelium. As previously reported by others, we find that during mid-oogenesis, Cad99C localises specifically to the apical domain of follicle cells that are initiating biogenesis of apical microvilli (D’Alterio et al., 2005; Schlichting et al., 2006). These Cad99C positive microvilli are visible by staining for filamentous actin (F-actin) or by transmission electron microscopy (TEM) (Fig 1A-D). Interestingly, we also see Rab11 endosomes localising apically in follicle cells around the time of microvilli biogenesis (Fig 1E,F). Since Rab11 endosomes are known to be involved in endocytic recycling to the apical membrane, as well as in trans-Golgi to plasma membrane exocytic delivery (Jing and Prekeris, 2009; Rodriguez-Boulan and Macara, 2014) and microvillus formation in enterocytes (Knowles et al., 2015), we examined their role in trafficking Cad99C to the apical membrane by inducing Rab11 RNAi in follicle cells. Knockdown of Rab11 results in loss of Cad99C from the apical membrane, suggesting that trafficking of Cad99C occurs via Rab11 endosomal transport (Fig 1G). To rule out an indirect effect of Rab11 on Cad99C trafficking via misregulation of epithelial polarity, we tested the effect of Rab11 RNAi on markers of cell polarity. We find that localisation of aPKC and Dlg is not affected upon Rab11 knockdown (supplementary Fig 1A). These results show that epithelial polarity is retained in Rab11 RNAi cells.

Rab11 endosomes use an array of adaptor proteins to bind to different motors for intracellular transport (Horgan and McCaffrey, 2009; Junutula et al., 2004; Meyers and Prekeris, 2002; Prekeris, 2003). Nuclear fallout (Nuf) has previously been shown to interact with Rab11 and be required for its correct localisation to the cleavage furrow during cytokinesis (Cao et al., 2008; Riggs et al., 2003). Nuf is also known to directly interact with the minus-end motor Dynein to transport cargo towards microtubule minus-ends (Riggs et al., 2007). We find that Nuf localises apically in follicle cells (Fig 1H). We therefore tested the requirement for Nuf and Dynein to transport Rab11 endosomes. nuf mutants and knockdown of Dynein both reveal mislocalisation of Rab11 endosomes from the apical membrane to the cytoplasm (Fig 1I). These results demonstrate the importance of Nuf and Dynein for correct apical localisation of Rab11 endosomes in the follicle cell epithelium. We find that cell polarity is not affected in nuf mutants or Dynein RNAi follicle cells, as aPKC and Dlg are localised normally in both conditions (supplementary Fig 1A, B).
We next studied the requirement for microtubules in Rab11 endosome trafficking and microvilli biogenesis. We induced overexpression of the microtubule-severing protein Katanin-60 to trigger depolymerisation of most microtubules (Diaz-Valencia et al., 2011), and find that loss of microtubules leads to accumulation of Rab11 endosomes in the cytoplasm and failure of Cad99C delivery (Fig 2A,B). We also depolymerised the microtubules in follicle cells by treating the egg chambers with colchicine for 1-hour. Control egg chambers had apical Rab11 localisation, whereas egg chambers treated with colchicine had endosomes accumulating basally in the follicle cells (Fig 2C,D). Thus, the polarisation of Rab11 endosomes for delivery of Cad99C is a microtubule-dependent process.

To explore how the microtubules become polarised in follicle cells, we considered the roles of two microtubule-binding proteins Patronin (CAMSAP1/2/3 in humans) and Shot (MACF1/BPAG1 in humans). Patronin has been reported to bind minus-ends of microtubules via its C-terminal CKK domain and protect them from Kinesin-13 mediated degradation (Baines et al., 2009; Goodwin and Vale, 2010; Hendershott and Vale, 2014). Furthermore, in mammalian cells CAMSAP3 and CAMSAP2 have been shown to cooperate to organise epithelial-specific organisation of acentrosomal microtubules (Tanaka et al., 2012; Toya et al., 2016). Shot is a spectraplakin cytoskeletal protein, known to crosslink microtubules to the actin cytoskeleton (Applewhite et al., 2010; Lee and Kolodziej, 2002). Shot can bind F-actin via its N-terminal actin binding domain and microtubules via its C-terminal GAS2 domain (Applewhite et al., 2010; Lee and Kolodziej, 2002; Lee et al., 2000; Sun et al., 2001).

We show that GFP-tagged Patronin and Shot localise apically in follicle cells, suggesting a potential role in polarising the microtubule cytoskeleton along the apical-basal axis of epithelial cells (Fig 2E,F).

We depleted Patronin in follicle cells by RNAi, which produces a moderately disordered microtubule cytoskeleton, mildly affecting Rab11 trafficking (Fig 2G). Cad99C localisation remains largely unaffected, likely due to a slow turnover rate of the protein (Fig 2G). We next investigated the requirement of Shot using shotg null mutants, which has previously been reported to cause occasional double layering in the follicle cell epithelium (Gregory and Brown, 1998; Roper and Brown, 2003). Mutants of shotg moderately affect microtubule polarisation and Rab11 endosome trafficking, with minimum effect on Cad99C localisation (Fig 2H). Due to the weak phenotypes of losing Patronin and Shot individually, we combined the two manipulations to see whether this causes a stronger phenotype. We find that
perturbing both proteins severely affects microtubule organisation and leads to loss of Cad99C from the apical membrane (Fig. 2I-L). We find that severe disruption of microtubule organisation often leads to mis-positioning of nuclei in follicle cells, giving cells the appearance of multilayering, when they are actually still a monolayer. Our results indicate that Patronin and Shot work in parallel to polarise microtubules, and that microtubule polarisation is essential for apical delivery of Cad99C in follicle cells.

We next investigated the mechanism by which Patronin and Shot become polarised to the apical domain. We considered the role of Spectrins in polarising Patronin and Shot to the apical membrane. The Spectrin cytoskeleton is polarised in epithelial cells with α2β12 heterotetramers localising to the apical domain and α2β6 heterotetramers localising to the basolateral domain (Thomas and Kiehart, 1994b; Thomas and Williams, 1999; Zarnescu and Thomas, 1999a). Several lines of evidence suggest that apical Spectrins interact with Patronin and Shot. Firstly, a conserved region in mammalian CAMSAP1, known as the CC1 region, has been shown to bind the linker region adjacent to the PH domain of the long C-terminal variant of βII-Spectrin in vitro (Fig. 3A) (King et al., 2014). Secondly, we identified α and βH-Spectrin/Karst in the mass spectrometry analysis of Patronin or Shot-associated proteins in Drosophila (data not shown). Finally, Shot contains multiple spectrin repeat domains, suggesting that it might directly bind to spectrins (Fig 3A) (Leung et al., 1999; Roper and Brown, 2003; Sun et al., 2001).

To test the requirement for the Spectrin cytoskeleton in localising Shot and Patronin, we induced mutant clones for α-spectrin in the follicle cell epithelium. We find that loss of α-Spectrin causes mislocalisation of Shot from the apical domain and also affects Rab11 and Cad99C localisation (Fig. 3 B-E). Our observation is not an indirect affect of loss of polarity, as aPKC is not affected in α-spectrin mutants (Fig 3E, supplementary Fig 2A). Although the apical polarity is maintained, we find that α-spectrin mutant cells appear to lose their perivitelline space and associate closely with the oocyte membrane, indicating that these cells may have defective microvilli (Fig 3E, bottom panel). Consistent with this finding, we show that loss of α-Spectrin prevents apical F-actin microvilli formation, but does not affect cortical F-actin in follicle cells (supplementary Fig 2B, C). We confirm that loss of α-Spectrin or β-Spectrin can also cause a reduction in cell height, as recently reported (supplementary Fig 2D-F) (Ng et al., 2016).
We find that mutation of βH-spectrin/karst or shot alone does not have a strong affect on Cad99C localisation (supplementary Fig 3A, B). Due to the similar structure and role of βH-spectrin/karst and Shot in binding microtubules, we anticipated that there might be redundancy between the two proteins. To test this possibility, we analysed Cad99C localisation in double mutants of shot and βH-spectrin/karst. Indeed, we find that Cad99C is lost from the apical membrane in the double mutants (Fig 3F, supplementary Fig 3C).

We next tested for interactions of Spectrins with Patronin and Shot by performing co-immunoprecipitation (Co-IP) experiments from Drosophila embryos expressing endogenously YFP-tagged βH-Spectrin/Karst. We find that Karst interacts strongly with two isoforms of Shot (Fig 3G). Pulling down Karst also co-immunoprecipitates endogenous α-Spectrin. We also performed Co-IP experiments in embryos expressing Shot-GFP and Patronin-GFP. We find that both Shot and Patronin bind to βH-Spectrin/Karst and α-Spectrin (Fig 3G). Furthermore, we find that Patronin can bind to Shot (Fig 3G). These results indicate that apical spectrins bind to Patronin and Shot, and act to recruit the two proteins to the apical membrane. Consistent with the data from the Co-IP experiments, we show that double mutants of shot and βH-spectrin/karst lose polarisation of Patronin from their apical domains (Fig 3H). In addition, these double mutants display severe defects in microtubule organisation (Fig 3I), further supporting the notion that Shot and βH-Spectrin/Karst act redundantly to polarise microtubules in the follicle cell epithelium.

In epithelial cells, fundamental determinants of apical-basal cell polarity are responsible for polarising all other proteins in the cell. We sought to determine whether two key apical and basal polarity determinants, Cdc42 and Lgl, were important to organise polarisation of apical Spectrins to direct the polarisation of downstream trafficking machinery for Cad99C. We find that mutants of cdc42 and lgl exhibit mislocalisation of apical Spectrins, Patronin and Shot, and also exhibit loss of Cad99C from the apical membrane (supplementary Fig 4A-I). The loss of Cdc42 or Lgl causes a dramatic disruption of the epithelial tissue, making it difficult to determine whether these determinants act directly or indirectly to polarise Spectrins, Patronin and Shot. Nevertheless, these findings suggest that apical-basal polarity determinants act upstream of Spectrin polarisation to control Patronin and Shot localisation and microtubule polarisation, which then directs apical trafficking of Cad99C for microvilli biogenesis (Fig 3J).
Once Rab11 endosomes are transported apically along microtubules by the Nuf-Dynein motor complex, they must traverse the apical F-actin cortex to be delivered to the plasma membrane. We find that a different motor complex is required to transport the endosomes beyond the microtubule network. Myosin V (MyoV or MyoVa/b in humans) is a known actin-based motor, implicated in polarised membrane transport of Rab11 endosomes in both mammals and flies (Lapierre et al., 2001; Li et al., 2007). Additionally, the Drosophila Rab11-interacting protein (dRip11, known as Rab11FIP1 in humans) has also been shown to bind Rab11 endosomes, as well as interact in a complex with and MyoV during Rhodopsin transport in developing photoreceptors in Drosophila (Li et al., 2007; Prekeris et al., 2000). Based on these interactions, we investigated the roles of MyoV and dRip11 in apical delivery of Rab11 endosomes using dominant negative lines of both proteins, dRip11-CT-GFP and MyoV-CT-GFP, which express a C-terminal GFP-tagged version of the proteins (Li et al., 2007). Expression of dRip11-CT-GFP and Myo-CT-GFP caused accumulation of Rab11 and Cad99C in the sub-apical region of follicle cells, with MyoV causing a more severe effect (Fig 4A-C). We find that Rab11 co-localises with Cad99C in these accumulated endosomes (Fig 4B, C). We show that disrupting the microtubules with Colchicine in follicle cells expressing Myo-CT-GFP causes the accumulated endosomes to redistribute basally (Fig 4D), which is reminiscent of the basal endosomes found in Dynein RNAi cells (Fig 4E), where Rab11 and Cad99C also co-localise. These results suggest that dRip11 and MyoV are dispensable for apical transport of Rab11 endosomes along microtubules but are required for their apical delivery through the F-actin cortex to the plasma membrane (Fig 4F).
Discussion

Our results reveal a mechanism linking determinants of cell polarity with stepwise polarisation of the spectrin cytoskeleton, microtubule cytoskeleton and biogenesis of actin microvilli via apical trafficking of Cad99C. The results suggest that polarisation of the apical spectrin, βH-Spectrin/Karst, is dependent on polarity determinants, likely via interactions with the FERM domain proteins and the apical polarity determinant Crb (Fletcher et al., 2015; Medina et al., 2002). The spectraplakin Shot is highly similar to βH-Spectrin/Karst, and is able to bind to and co-localise with it at the apical domain of epithelial cells, suggesting that the two proteins might have a similar function. βH-Spectrin/Karst is linked to microtubules via Patronin, while Shot can directly bind microtubules. Consequently, redundancy is anticipated between βH-Spectrin/Karst and Shot, or between Patronin and Shot. Accordingly, we find that mutation of βH-spectrin/karst only has a mild phenotype, while mutation of α-spectrin simultaneously disrupts both pairs of proteins in parallel and causes a drastic phenotype, completely disrupting the apical trafficking of Cad99C and microvillar biogenesis. More importantly, double mutants for shot and βH-spectrin/karst have a severe effect on microtubule and Cad99C localisation than either alone, therefore demonstrating that the two proteins act in a redundant fashion.

Downstream of the Spectrin cytoskeleton, Patronin and Shot are required in parallel to drive apical-basal polarisation of microtubules, which are then responsible for orienting the apical transport of Cad99C, within Rab11 endosomes, by the Dynein motor protein. Eliminating microtubules from cells by overexpressing Katanin60 results in loss of Nuf-Dynein-based apical Rab11 endosome transport and failure to efficiently deliver Cad99C to the apical membrane. The effect on Cad99C polarisation is not an indirect effect of loss of polarity due to impaired Rab11 and Dynein function in localising the apical polarity determinant Crumbs to the apical membrane (Horne-Badovinac and Bilder, 2008; Li et al., 2008) because firstly, polarity is maintained in cells expressing Rab11 or Dynein RNAi, as indicated by the normal localisation of aPKC and secondly, loss of Crb does not strongly affect cell polarity in the follicle cell epithelium due to redundancy with Bazooka (Fletcher et al., 2012b). Our results indicate that even under conditions with severe depletion of microtubules, the overall shape of the follicle cell epithelium is relatively normal, indicating that polarised microtubules are required to influence formation of apical microvilli, rather than for other functions of the actin cytoskeleton in epithelial cells. Similarly, we do not see strong effects on cell shape upon loss of either Patronin or
Shot (or both), raising questions over the claimed requirement for Patronin homologs and microtubules in formation or maintenance of adherens junctions epithelial cells in culture (Chen et al., 2003; Le Droguen et al., 2015; Meng et al., 2008; Stehbens et al., 2006).

The final step in delivery of Cad99C to the apical membrane also requires actin-based transport via the action of dRip11-MyoV complex. Compromising normal MyoV function in *Drosophila* follicle cells by expressing a dominant negative version of the protein, results in loss of Rab11 polarisation from the apical membrane and its abnormal accumulation in the sub-apical region. This phenotype in *Drosophila* shows similarities with the human microvillus inclusion disease, where mutations in MyoVb gene also cause loss of Rab11 endosomes from the apical membrane (Knowles et al., 2014; Lapierre et al., 2001).

In summary, our results reveal how the Spectrin cytoskeleton acts to polarise microtubules in epithelial cells, and how polarised microtubules then direct trafficking of Rab11 endosomes carrying Cad99C to the apical membrane. This process relies on a hierarchy of events, and disruption at any stage can lead to failure in delivering Cad99C to the apical membrane, resulting in defective biogenesis of microvilli. Our findings are directly relevant to human diseases such as Usher’s Syndrome Type 1 and microvillus inclusion disease, helping to outline the molecular and cellular basis for these conditions.
Materials and Methods

Mitotic clones in follicle cells were generated using the FLP/FRT site-specific recombination system and were either marked negatively (absence of GFP) or positively (presence of GFP; MARCM) (Lee and Luo, 1999; Xu and Rubin, 1993). Newly eclosed females were heat-shocked once at 37°C for 1 hour and ovaries were dissected 5 days after heat-shock.

The “Flip-out” actin.FRT.CD2.FRT.Gal4/UAS system was used to express the UAS-Rab11IR construct. To express the transgenes, newly eclosed females were heat-shocked at 37°C for 10 minutes and ovaries were dissected 2 days after heat-shock. Expression of other UAS-driven transgenes in follicle cells was achieved with the follicle cell specific Gal4 drivers GR1.Gal4 and Traffic Jam.Gal4 (Tj.Gal4), as well as the MARCM system. w or flies were used as the “wild-type” stock.

Fly stocks

RNAi lines were ordered from the Vienna Drosophila Resource Center: Patronin IR (VDRC 27654) and Dynein IR (VDRC 28054). Rab11 RNAi line was generated by R. Brain in the lab. UAS.shot-GFP, FRT42B shot\(^5\), Ubi.patronin-GFP, UAS.katanin60, GR1.Gal4, FRT19A cdc42\(^3\) and FRT40A lgl\(^4\) were ordered from Bloomington Drosophila Stock Center. UAS.myoV-CT-GFP and UAS.dRip11-CT-GFP were gifts from D. Ready (Li et al., 2007). Rab11-YFP was a gift from M. Brankatschk. Kst-YFP (DGRC 115-285), Tj.Gal4 (DGRC 104-055) and FRT80B nuf (DGRC 111-536) were ordered from Drosophila Genetic Resource Center, Kyoto. The following strains were used from previous studies: \(\alpha\)-spec\(^{226}\) (Huelsmeier et al., 2007), kst\(^{d1113}\) (Campos et al., 2010), kst\(^1\) (Thomas and Kiehart, 1995), Nod.lacZ and Kin.lacZ (Clark et al., 1997).

Drosophila genotypes

Fig 1B w
Fig 1C w
Fig 1D w
Fig 1E w
Fig 1F w;; Rab11-YFP
Fig 1G yw hsflp/+; actin.FRT.CD2.FRT.Gal4/+; UAS.Rab11-IR/UAS.GFP
Fig 1H w
Fig 1I yw hsflp tub.Gal4 UAS.GFPnls/+;; FRT80B tubG80/FRT80B
yw hsflp tub.Gal4 UAS.GFPnls/+; FRT80B tubG80/nuf FRT80B
yw hsflp/+; UAS.CD8-GFP/+; GR1.Gal4/+ 
w;; GR1.Gal4/ UAS.dynein-IR

Fig 2A yw hsflp/+; UAS.CD8-GFP/+; GR1.Gal4/+ 
Fig 2B w;; GR1.Gal4/ UAS.katanin60 
Fig 2C w 
Fig 2D w 
Fig 2E w;; GR1.Gal4/ ubi.patronin-GFP 
Fig 2F w;; GR1.Gal4/ UAS.shot-GFP 
Fig 2G w; UAS.patronin-IR/+; GR1.Gal4/+ 
Fig 2H yw hsflp tub.Gal4 UAS.GFPnls/+; FRT42B tubG80/shot³ FRT42B 
Fig 2I yw hsflp tub.Gal4 UAS.GFPnls/+; FRT42B tubG80/shot³ FRT42B, UAS.patronin-IR 
Fig 2J yw hsflp/+; UAS.CD8-GFP/+; GR1.Gal4/+ 
Fig 2K yw hsflp tub.Gal4 UAS.GFPnls/+; FRT42B tubG80/shot³ FRT42B, UAS.patronin-IR 
Fig 2L yw hsflp tub.Gal4 UAS.GFPnls/+; FRT42B tubG80/shot³ FRT42B, UAS.patroninIR

Fig 3B yw hsflp tub.Gal4 UAS.GFPnls/+; FRT80B tubG80/FRT80B 
Fig 3C yw hsflp tub.Gal4 UAS.GFPnls/+; FRT80B tubG80/α-spectrinε226 FRT80B 
Fig 3D yw hsflp tub.Gal4 UAS.GFPnls/+; FRT80B tubG80/α-spectrinε226 FRT80B 
Fig 3E yw hsflp/+; α-spectrinε226 FRT80B/ubiGFPnls FRT80B 
Fig 3F yw hsflp/+; shot³ FRT42B/ubiGFPnls FRT42B; kst¹ FRT80B/ubiGFPnls FRT80B 
Fig 3H yw hsflp/+; shot³ FRT42B/ubiGFPnls FRT42B; kst¹ FRT80B/ubiGFPnls FRT80B 
Fig 3I yw hsflp/+; shot³ FRT42B/ubiGFPnls FRT42B; kst¹ FRT80B/ubiGFPnls FRT80B 
Fig 3J w 
w;; GR1.Gal4/ ubi.patronin-GFP 
w;; GR1.Gal4/ UAS.shot-GFP 

Fig 4A yw hsflp/+; UAS.CD8-GFP/Tj.Gal4 
Fig 4B yw hsflp/+; Tj.Gal4/++; UAS.dRip11-CT-GFP 
Fig 4C yw hsflp/+; Tj.Gal4/++; UAS.myoV-CT-GFP
Fig 4D yw hsflp/+; Tj.Gal4/+; UAS.myoV-CT-GFP
Fig 4E w+; GR1.Gal4/ UAS.dynein-IR

Supplementary Figures

Supp Fig 1A  yw hsflp tub.Gal4 UAS.GFPnls/+;; FRT80B tubG80/FRT80B
yw hsflp tub.Gal4 UAS.GFPnls/+;; FRT80B tubG80/nuf FRT80B
yw hsflp/+; actin.FRT.CD2.FRT.Gal4/+; UAS.Rab11-IR/UAS.GFP

Supp Fig 1B  yw hsflp/+; UAS.CD8-GFP/+; GR1.Gal4/+;
w+; GR1.Gal4/ UAS.dynein-IR

Supp Fig 2A  yw hsflp/+;; α-spectrin^626 FRT80B/ubiGFPnls FRT80B
Supp Fig 2B  yw hsflp/+;; α-spectrin^626 FRT80B/ubiGFPnls FRT80B
Supp Fig 2C  yw hsflp/+;; α-spectrin^626 FRT80B/ubiGFPnls FRT80B
Supp Fig 2D  yw hsflp/+;; α-spectrin^626 FRT80B/ubiGFPnls FRT80B
Supp Fig 2E  β-spectrin^6113 FRT19A/ubiRFPnls FRT19A; hsflp/+;
Supp Fig 2F  β-spectrin^6113 FRT19A/ubiRFPnls FRT19A; hsflp/+;

Supp Fig 3A  yw hsflp/+; shot^3 FRT42B/ubiGFPnls FRT42B; +/ubiGFPnls FRT80B
Supp Fig 3Byw hsflp/+; +/ubiGFPnls FRT42B; kst^1 FRT80B/ubiGFPnls FRT80B
Supp Fig 3C  yw hsflp/+; shot^3 FRT42B/ubiGFPnls FRT42B; kst^1 FRT80B/ubiGFPnls FRT80B
Supp Fig 3D  yw hsflp tub.Gal4 UAS.GFPnls/+; FRT80B tubG80/kst^6113 FRT80B

Supp Fig 4A  yw hsflp FRT19A tubG80/FRT19A;; tub.Gal4 UAS.GFP/+;
Supp Fig 4B  yw hsflp FRT19A tubG80/cdc42^3 FRT19A;; tub.Gal4 UAS.GFP/+;
Supp Fig 4C  yw hsflp tub.Gal4 UAS.GFPnls/+; FRT40A tubG80/ig^4 FRT40A
Supp Fig 4D  yw hsflp FRT19A tubG80/FRT19A; ubi.patronin-GFP/+; tub.Gal4/+;
Supp Fig 4E  yw hsflp FRT19A tubG80/cdc42^3 FRT19A; ubi.patronin-GFP/+; tub.Gal4/+;
Supp Fig 4F  yw hsflp/+; FRT40A tubG80/ig^4 FRT40A; ubi.patronin-GFP/tub.Gal4
Supp Fig 4G  yw hsflp FRT19A tubG80/FRT19A;; tub.Gal4 UAS.GFP/+;
Supp Fig 4H  yw hsflp FRT19A tubG80/cdc42^3 FRT19A;; tub.Gal4 UAS.GFP/+;
Supp Fig 4I  yw hsflp tub.Gal4 UAS.GFPnls/+; FRT40A tubG80/ig^4 FRT40A
Supp Fig 4J  yw hsflp tub.Gal4 UAS.GFPnls/+; FRT82B tubG80/ FRT82B
Supp Fig 4K  yw hsflp tub.Gal4 UAS.GFPnls/+; FRT82B tubG80/ FRT82B crb^11A22
Immunostaining of ovaries and microscopy

Ovaries were dissected in PBS, fixed for 20 minutes in 4% paraformaldehyde in PBS, washed for 30 minutes in PBS/0.1% Triton X-100 (PBST) and blocked for 30 minutes in 5% normal goat serum/PBST (PBST/NGS). Primary antibodies were diluted in PBST/NGS and samples were incubated overnight at 4°C.

For Crumbs staining, ovaries were fixed for 10 minutes in 8% paraformaldehyde in PBS, washed in methanol for 5 minutes, washed for 20 minutes x 3 in PBST, washed for 5 minutes in 1% SDS, rinsed in PBS x 3 and blocked for 30 minutes in 5% PBST/NGS. The rest of the staining was carried out as mentioned before.

Primary antibodies used were: rabbit anti-aPKC (C20, Santa Cruz), mouse anti-Dlg (4F3, DSHB), mouse anti-α-Spectrin (3A9, DSHB), rabbit anti-βH-Spectrin (G. Thomas), rabbit anti-Cad99C (C. Dahmann), guinea pig anti-Cad99C (D. Godt), guinea pig anti-Shot (K. Roper), rabbit anti-Rab11 (A. Nakamura), mouse anti-Crumbs (CQ4, DSHB), mouse anti-α-tubulin (Sigma) and rabbit anti-Nuf (S. Hayashi). Phalloidin-TRITC (Sigma) was used to stain F-actin. Secondary antibodies (all from Molecular Probes, Invitrogen) were used at 1:500 for 2 hours at room temperature along with DAPI staining at 1 μg/ml and then washed multiple times in PBST. Samples were mounted on slides in Vectashield (Vector labs). Images were acquired on a Zeiss LSM710 confocal microscope using 40x or 63x oil immersion objectives, and processed using Adobe Photoshop. Optical cross-sections through the middle of egg chambers are shown in all figures.

Colchicine treatment

Wild type egg chambers were cultured in imaging media containing Schneider’s Media (Invitrogen), Insulin (Sigma), heat-inactivated FCS (GE Healthcare), Trehalose (Sigma), Adenosine Deaminase (Roche), Methoprene (Sigma) and Ecdysone (Sigma) (Prasad et al., 2007), with 0.2mg/ml of Colchicine or Ethanol (for control) for 1 hour at room temperature. After treatment, samples were fixed and processed normally for imaging.

Co-immunoprecipitation

For Co-IP experiments, Drosophila Karst YFP knock-in embryos (DGRC 115285), Wiso embryos, and embryos expressing Patronin-GFP or Shot-GFP were collected over 24 h at 22°C before being lysed in buffer containing 10 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40 and 0.5 mM EDTA (Chromotek), plus PhosSTOP Phosphatase
Inhibitor Cocktail Tablets (Roche), protease inhibitor cocktail (Roche), 0.1 M NaF and 1 mM PMSF. Samples were left on ice to solubilise for 30 min, before being centrifuged at high speed (14,000 rpm for 30 min at 4°C). The supernatant was collected, pre-cleared and incubated with GFP Trap-M beads (Chromotek).

Western blots were probed with mouse anti-GFP (Roche), guinea pig anti-Shot (K. Roper), rabbit anti-Patronin (R. Vale), mouse anti-α-Spectrin (3A9, DSHB) and rabbit anti-βH-Spectrin (G. Thomas) antibodies, before being detected with chemiluminescence (GE Healthcare).

**Electron microscopy of Drosophila egg chambers**

Drosophila egg chambers were fixed in 2.5% glutaraldehyde and 4% formaldehyde in 0.1M phosphate buffer (pH 7.4) and then processed for transmission electron microscopy (TEM) and serial block-face scanning electron microscopy (SBFSEM). Samples were prepared using the National Center for Microscopy and Imaging Research (NCMIR) method (Deerinck et al., 2010). For TEM, 70nm sections were cut using a UCT ultramicrotome (Leica Microsystems) and collected on formvar coated slot grids. No post-staining was required due to the density of metal deposited using the NCMIR protocol. Images were acquired using a 120 kV Tecnai G2 Spirit Biotwin (FEI Company) and Orius CCD camera (Gatan Inc.).
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Figure 1: Cad99C is trafficked to the apical membrane via Rab11 endosomes during microvilli morphogenesis

(A) Schematic diagram of a stage 10 Drosophila egg chamber, highlighting cells that make microvilli. (B) Wild type egg chambers at different stages of oogenesis stained for DAPI to mark nuclei and F-actin to visualise the apical, actin-rich microvilli. (C) Transmission electron microscopy images of wild type egg chambers at different stages of microvilli biogenesis. Arrows point to apical microvilli in follicle cells. Oc: oocyte, FC: follicle cells, V.B.: vitelline bodies. Cad99C (D) and Rab11 (E) become polarised apically during stages of microvilli biogenesis in wild type egg chambers. (F) Egg chambers expressing endogenous Rab11-YFP. (G) Expression of Rab11 RNAi (GFP positive clone) causes mislocalisation of Cad99C. (H) Nuf is localised apically during stages of microvilli biogenesis. (I-L) Mutation of nuf (GFP positive clone) or knockdown of Dynein (whole egg chamber) causes mislocalisation of Rab11 endosomes. (K) Zooms of images in (J).
Figure 2: Cad99C is transported apically along microtubules that are polarised by Patronin and Shot

(A) Control egg chamber stained for Cad99C, Rab11, DAPI and Tubulin to show polarised microtubules. (B) Overexpression of Katanin60 causes microtubules to depolymerise, resulting in loss of Rab11 and Cad99C polarisation. Control egg chambers (C) or egg chambers treated with Colchicine (D) to depolymerise microtubules; Rab11 polarisation is lost upon Colchicine treatment. Expression of Patronin-GFP (E) and Shot-GFP (F) shows both proteins localise apically. Expression of Patronin RNAi (G) or mutation of shot (GFP positive clone) (H) causes depolarised microtubules, affecting Rab11 localisation and Cad99C protein levels. (I-L) Combined perturbation of Patronin and Shot (GFP positive clone) results in loss of Cad99C from the apical membrane and causes severe defects in microtubule polarisation. Loss of polarised microtubules results in the mislocalisation of nuclei in these mutants, which gives the impression of multilayering of the follicle cells.
Figure 3: The spectrin cytoskeleton is required to polarise Patronin and Shot in response to apical-basal polarity determinants

(A) Schematic diagram of protein domain structures of Patronin, βH-Spectrin/Karst and Shot. (B) Control egg chamber stained for Shot and Cad99C. Mutation of α-spectrin (GFP positive clone) causes mislocalisation of Shot, Cad99C (C) and Rab11 (D). (E) Top panel: α-spectrin mutants (GFP negative clone) exhibit loss of Cad99C from the apical membrane. Bottom panel: α-spectrin mutants (GFP negative clone) have normal aPKC polarisation but show loss of perivitelline space between the follicle cell membrane and the oocyte membrane (arrow), suggesting microvilli defects. (F) Double mutants for Shot and βH-Spectrin/Karst (GFP negative clone) show loss of Cad99C from the apical membrane. (G) Left panel: Co-IP of endogenous Karst-YFP knock-in embryos with Shot and α-Spectrin. Middle panel: Co-IP of UAS.Shot-GFP embryos with βH-Spectrin/Karst and α-Spectrin. Right panel: Co-IP of Patronin-GFP embryos with βH-Spectrin/Karst, α-Spectrin and Shot. (H) Top panel: Double mutants for Shot and βH-Spectrin/Karst (GFP negative clone) exhibit loss of Patronin from the apical domain (arrow), bottom panel: Shot staining in Shot and βH-Spectrin/Karst double mutants. (I) Double mutants for Shot and βH-Spectrin/Karst (GFP negative clone) show severe defects in microtubule polarisation (arrow). (J) Stepwise representation of events leading to the polarisation of Cad99C at the apical membrane for biogenesis of microvilli.
Figure 4: The microvilli inclusion disease protein MyoV is required for apical delivery of Rab11 endosomes

(A) Control egg chamber expressing UAS.CD8GFP and stained for Cad99C and Rab11. Expression of dominant negative dRip11-CT-GFP (B) or MyoV-CT-GFP (C) causes accumulation of Rab11 endosomes and Cad99C (arrows) near the apical region. (D) Colchicine treatment of egg chambers expressing dominant negative MyoV-CT-GFP causes basal accumulation of Rab11 endosomes and Cad99C in follicle cells (arrows). (E) Basal accumulation of Rab11 endosomes and Cad99C also occurs in follicle cells expressing Dynein RNAi. (F) Model for normal trafficking and delivery of Cad99C to promote apical microvilli biogenesis. Defects in trafficking or delivery of Cad99C results in loss of Cad99C function and leads to diseases such as Usher syndrome Type 1 and microvillus inclusion disease.
References


Supp Fig 1: Trafficking of Rab11 endosomes via Nuf and Dynein does not affect polarisation of aPKC and Dlg

(A) Top and middle panels: aPKC and Dlg localisation in control and nuf mutant (GFP positive clone) follicle cells; cell polarity is maintained in nuf mutants. Bottom panel: Rab11 RNAi (GFP positive clone) does not affect aPKC localisation but specifically affects Cad99C localisation at the apical membrane of follicle cells.

(B) aPKC and Dlg localisation in control and Dynein RNAi follicle cells; polarity is not affected upon Dynein RNAi.
Supp Fig 2: The Spectrin cytoskeleton is not required to polarise aPKC and cortical F-actin, but is required for apical F-actin microvilli formation and columnar cell height

Loss of α-spectrin (GFP negative clone) does not affect aPKC localisation (A) or F-actin polarisation (B), however it does affect microvilli formation (arrows in C). Loss of α-spectrin (GFP negative clone) also causes a reduction in cell height, a phenotype not observed when microtubule polarisation is disrupted, suggesting that this is a separate function of the Spectrin cytoskeleton (D). In support of this notion, loss of β-spectrin (RFP negative clone) also causes a reduction in cell height and a reduction in the septate junction marker Coracle (Cora) (E,F).
Supp Fig 3: βH-Spectrin/Karst acts redundantly with Shot to polarise Cad99C

Single mutants of shot (A) or βH-spectrin/karst (B) (clones marked with one copy of GFP) do not have an effect on Cad99C localisation, while double mutants of shot and βH-spectrin/karst (GFP negative clone) exhibit loss of Cad99C from the apical membrane (C). (D) βH-spectrin/karst mutants (GFP positive clone) are not sufficient to cause mislocalisation or loss of Patronin from the apical domain.
Supp Fig 4: Core apical-basal polarity determinants, but not Crumbs, are essential to polarise the Spectrin cytoskeleton, Patronin, Shot and Cad99C

α-Spectrin and β1r-Spectrin/Karst localisation in control (A), cdc42 (B) or lgl (C) mutants (clones highlighted in box). Patronin and Shot localisation in control (D), cdc42 mutant (full clone; E) or lgl mutant (clones highlighted in box; F). Cad99C localisation in control (G), cdc42 (H) or lgl (I) mutants (GFP positive clone); the presence of apical domain in lgl mutant clones is not sufficient to polarise Cad99C to the apical membrane. Cad99C localisation is not affected in large null mutants for crb (GFP positive clone), consistent with the notion that Crb acts redundantly with Baz to organise apical-basal polarity in follicle cells (J, K).
Movie S1. EB1-GFP in the follicle cell epithelium.