Drosophila CK1-γ, Gilgamesh, controls PCP-mediated morphogenesis through regulation of vesicle trafficking

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Cellular morphogenesis, including polarized outgrowth, promotes tissue shape and function. Polarized vesicle trafficking has emerged as a fundamental mechanism by which protein and membrane can be targeted to discrete subcellular domains to promote localized protrusions. Frizzled (Fz)/planar cell polarity (PCP) signaling orchestrates cytoskeletal polarization and drives morphogenetic changes in such contexts as the vertebrate body axis and external Drosophila melanogaster tissues. Although regulation of Fz/PCP signaling via vesicle trafficking has been identified, the interplay between the vesicle trafficking machinery and downstream terminal PCP-directed processes is less established. In this paper, we show that Drosophila CK1-γ/Gilgamesh (gish) regulates the PCP-associated process of trichome formation through effects on Rab11-mediated vesicle recycling. Although the core Fz/PCP proteins dictate prehair formation broadly, CK1-γ/gish restricts nucleation to a single site. Moreover, CK1-γ/gish works in parallel with the Fz/PCP effector multiple wing hairs, which restricts prehair formation along the perpendicular axis to Gish. Our findings suggest that polarized Rab11-mediated vesicle trafficking regulated by CK1-γ is required for PCP-directed processes.

Introduction

Cellular morphogenesis requires polarized vesicle trafficking and cytoskeletal rearrangements to promote asymmetric shape changes (Lecuit, 2003). Diverse cell types rely on polarized protrusive activity to perform specialized functions. In yeast, polarized vesicle trafficking directs membrane deposition to discrete regions during budding and mating (Madden and Snyder, 1998). Directed cell migration and neurite outgrowth require polarized trafficking for asymmetric cytoskeletal accumulation and expansion at the leading edge (Zhou and Cohan, 2004; Heasman and Ridley, 2008; Fletcher and Rappoport, 2009). Furthermore, ciliogenesis requires a polarized cytoskeleton and vesicle trafficking (Nachury et al., 2007; Yoshimura et al., 2007). Lastly, planar polarized cytoskeletal protrusions are required for the elongation of the body axis in convergent extension (Shih and Keller, 1992; Wallingford et al., 2002).

Frizzled (Fz)/planar cell polarity (PCP) signaling promotes the orientation of cells in vertebrate and invertebrate tissues (Adler, 2002; Klein and Mlodzik, 2005; Lawrence et al., 2007; Wang and Nathans, 2007; Wu and Mlodzik, 2009). In Drosophila melanogaster, Fz/PCP signaling controls the formation of single, distally oriented actin-based hairs (trichomes) on each wing cell (Wong and Adler, 1993). The core group of Fz/PCP proteins, including membrane-associated Fz, Flamingo (Fmi), and Strabismus (Stbm)/Van Gogh and cytoplasmic Dishevelled (Dsh), Diego, and Prickle, localize asymmetrically during the formation of polarized cells (Axelrod, 2001; Das et al., 2002; Strutt et al., 2002; Jenny et al., 2003; Rawls and Wolff, 2003; Das et al., 2004).

In the wing, a bias in Fz transport along microtubules has been proposed as a mechanism for asymmetric polarization and distal trichome placement along the proximodistal (PD) axis (Shimada et al., 2006; Harumoto et al., 2010). Several PCP signaling effectors restrict trichome number downstream of the core PCP proteins, including Drosophila rho-associated kinase
(Drok), inturned, fuzzy, and multiple wing hairs (mwh; Wong and Adler, 1993; Winter et al., 2001; Strutt and Warrington, 2008; Yan et al., 2008). However, the precise mechanism by which these effectors interact and coordinate single trichome formation with other cellular components is not well understood.

From a PCP modifier screen, we have identified and characterized the function of the Drosophila casein kinase 1 γ (CK1-γ) homologue gilgamesh (gish) in the regulation of trichome morphogenesis. Genetic analysis in Drosophila has revealed a role for CK1-γ/gish in glial cell migration (Hummel et al., 2002), olfactory learning (Tan et al., 2010), and sperm individualization, a process requiring membrane remodeling (Nerusheva et al., 2009). CK1-γ/gish is evolutionarily conserved, and the redundant yeast orthologues YCK1/YCK2 are essential for bud site selection, morphogenesis, and cytokinesis (Wang et al., 1992; Robinson et al., 1993). GFP-Yck2p localizes to sites of polarized bud growth (Lew and Reed, 1995; Robinson et al., 1999). CK1-γ requires C-terminal palmitoylation for membrane localization and function (Robinson et al., 1993; Vancura et al., 1994; Davidson et al., 2005). Therefore, evidence from yeast budding and Drosophila spermatogenesis suggests that CK1-γ/gish may regulate polarized membrane trafficking.

Here, we describe a function for CK1-γ/gish in the regulation of PCP-directed morphogenesis. We find that CK1-γ/gish and the PCP effector mwh cooperate in an independent manner to restrict trichome formation to a single cell site. CK1-γ/gish regulates Rab11-mediated polarized vesicle trafficking. In animal cells, Rab11 localizes to recycling endosomes and derived vesicles, where it regulates vesicle trafficking during such processes as cell migration (Mammoto et al., 1999; Jing et al., 2009) and cell polarization (Prekeris et al., 2000; Wang et al., 2000; Pelissier et al., 2003). We also find that CK1-γ/gish regulates the localization of Rab11 effectors nuclear fallout (nuf; Rab11-FIP3 homologue) and Sec15 (Riggs et al., 2003; Zhang et al., 2004; Langevin et al., 2005). nuf and Rab11 are mutually required for localization to the recycling endosome (Riggs et al., 2003), and nuf is required for microtubule-dependent trafficking of recycling endosomes (Riggs et al., 2007; Horgan et al., 2010). Sec15, a component of the exocyst complex, is associated with secretory vesicles and is required for polarized exocytosis (TerBush et al., 1996). In summary, our data support a model by which CK1-γ/gish regulates morphogenesis in development through the spatial control of polarized vesicle trafficking.

**Results**

**gish, Drosophila CK1-γ, regulates cell and tissue morphogenesis**

To identify genes involved in PCP establishment, we performed a genetic modifier screen using the DrosDel deficiency collection (unpublished data; Ryder et al., 2007). Overlapping deficiencies defined a region on chromosome 3R that enhanced the PCP defects (Materials and methods and unpublished data). Subsequent analysis using upstream activation sequence (UAS)-RNAi (IR) identified gish (unpublished data), the Drosophila CK1-γ homologue (Hummel et al., 2002).

Expression of gishIR under the control of engtured (en)-Gal4 (Fig. 1, B compare with A) or FLP-FRT–induced gish01759 (strong hypomorphic allele; Jia et al., 2005) mutant clones (Fig. 1 C) produced similar trichome defects. These results were confirmed with an independent RNAi sequence, gishIR (also RNAi knockdown of the GFP fusion allele gishGFP was observed by en-Gal4 UAS-gishIR; Fig. S1, A [diagram] and B–C′′; Morin et al., 2001; Frescas et al., 2006).

To corroborate these results and determine the requirement of CK1-γ/Gish kinase activity, rescue experiments were performed. en-Gal4 expression of the Myc-GishWT transgene exhibited no phenotype (unpublished data), whereas its co-expression with gishIR partially rescued the trichome phenotype (Fig. S1, D, E and H, quantification). Two independent Myc-GishKD lines (Fig. S1 A, diagram for D187N kinase-dead mutation) failed to rescue the gishIR phenotype (Fig. S1, F and H, quantification; and not depicted). Myc-GishAC, a C-terminal truncation lacking the conserved palmitoylation site (Fig. S1 A), was ubiquitously localized and also failed to rescue the gishIR phenotype (Fig. S1, G and H, quantification). These data indicated that CK1-γ/Gish kinase activity and membrane association are required for trichome restriction.

Furthermore, to determine a general tissue requirement of CK1-γ/gish within the context of PCP, we analyzed whether trichome formation in the thorax results in the PCP morphogenetic process of ommatidial rotation in the eye. In the thorax, pannier (prn)-Gal4 expression of gishIR (Fig. 1, E compare with D) induced a phenotype with most cells projecting multiple trichomes (fz; or mwh loss in the thorax and trichome orientation and morphogenesis defects; Krasnow and Adler, 1994; Olguin et al., 2011). Expression of gishIR in photoreceptors under the control of the sevenless (sev)-Gal4 promoter or FLP-FRT–gish01759 clones induced misrotated ommatidial clusters (Fig. 1, G and H compare with F).

**CK1-γ/gish restricts trichome formation by focusing actin nucleation**

Just before prehair formation at 26–28 h after puparium formation (APF), much of the apical cell cortex consists of polymerized actin filaments (Eaton, 1997). At this stage, we observed no defects in actin polymerization in gishIR clones when compared with adjacent wild-type tissue (Fig. 2, A and A′). At 30–32 h APF, trichome formation appears at distal cell vertices as single focused actin-rich domains in wild-type tissue (Eaton, 1997). These domains often displayed multiple nucleation centers in gishIR clones (Fig. 2, B and B′, yellow arrowheads), indicating the primary defect in trichome morphogenesis occurred at this stage. These data suggested that the CK1-γ/gish loss-of-function (LOF) phenotype stemmed from a failure to restrict actin bundle nucleation to a single initiation site within the distal cell region. This was supported by later analysis (32–34 h APF), in which elongated trichomes displayed completely separate actin structures in CK1-γ/gish mutant cells (Fig. 2, C and C′, yellow arrowheads). This defect was observed in FLP-FRT gish01759 clones (Fig. S3, A and A′). This was also confirmed by transmission EM (TEM) on pupal wings expressing gishIR by nubbin (nub)-Gal4, which
Figure 1. CK1-γ/Gish regulates PCP-directed morphogenetic processes. (A–C) Wild-type and gish LOF wings. All images show adult wings oriented with distal to the right. (A) Control, engrailed (en)-Gal4, displaying single distally oriented trichomes. (B and C) en-Gal4 UASgishΔ (B) and FRT82-gish01739 clones (C) project multiple distally oriented trichomes. (D and E) Wild-type and gish LOF nota (dorsal thorax). Images show nota oriented with anterior to the top. (D) Control, pannier (pnr)-Gal4 notum, exhibits single posterior-oriented trichomes. (E) Many cells in pnr-Gal4 UAS-gishΔ tissue project multiple trichomes. (F–H) Wild-type and gish mutant adult eye sections. (F) Homozygous sevenless (sev)-Gal4 (sev-Gal4 2×) eyes display wild-type ommatidial orientation. (G and H) Homozygous sev>gishΔ (sev>gishΔ 2×; G) and gish01739 clones (H) exhibit misrotated ommatidia. Anterior is to the left. Diagrams on the bottom depict rhabdomere orientation; circles represent irregular photoreceptor number. Bars: (A–C) 25 µm; (D and E) 50 µm; (F–H) 10 µm.
revealed prehair separation to the apical cell surface (Fig. 2 D, red arrows).

Before prehair nucleation in wing cells, PCP proteins become asymmetrically localized through their interactions within the PD axis (Strutt and Strutt, 2009). Accordingly, mutations in core PCP genes result in symmetrical localization of the other PCP proteins (Strutt and Strutt, 2009). To determine whether CK1-γ/gish was required for PCP protein localization, we examined Fmi in gishIR tissue and gish01739 mutant clones. Asymmetric Fmi localization was not affected (Fig. 2, A′′ and B′′; and Fig. S3, A and compare C and C′ with B and B′). Collectively, our data support the hypothesis that CK1-γ/gish limits nucleation to a single location at the distal cell vertex through a mechanism downstream or in parallel to the core PCP components.

Interestingly, an increase in apical cell area was also observed in gishIR tissue (Fig. S2, A–B′), compare green FLP-out clone with adjacent tissue). Measurement of the apical area revealed a 60% increase relative to the adjacent wild-type cells (Fig. S2, B–C), whereas multiple prehair nucleation sites can be observed at 30–32 h APF (B and B′, yellow arrowheads). (A′′ and B′′) Fmi localization is asymmetric in gishIR tissue. (C–D) 32–34 h APF, independently nucleated actin prehairs are observed (C′, yellow arrowheads) and shown in a representative nubbin (nub)-Gal4 UAS-gishIR TEM section (D, red arrows indicate apical cell surface). Bars: (A–C) 10 µm; (D) 1 µm.

Figure 2. Gish regulates single trichome nucleation. (A–B′′) Pupal wing actin-Gal4 FLP-out clones expressing UAS-gishIR (blue; yellow lines mark clone border) stained with rhodamine-phalloidin (red) and Fmi. (A, A′, B, and B′) No defects in actin polymerization were observed from 26 to 28 h APF (A and A′), whereas multiple prehair nucleation sites can be observed at 30–32 h APF (B and B′, yellow arrowheads). (A′′ and B′′) Fmi localization is asymmetric in gishIR tissue. (C–D) 32–34 h APF, independently nucleated actin prehairs are observed (C′, yellow arrowheads) and shown in a representative nubbin (nub)-Gal4 UAS-gishIR TEM section (D, red arrows indicate apical cell surface). Bars: (A–C) 10 µm; (D) 1 µm.
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morphogenesis (Fig. S2, H and H)$^\text{II}$ or asymmetric Fmi localization (Fig. S2, H$^\text{II}$). Overall, these data suggest a direct role for CK1-γ/Gish in regulating trichome formation.

**CK1-γ/Gish localization during trichome morphogenesis**

The CK1-γ/Gish protein was associated with the base of developing prehairs as observed in apical confocal projections of 32–34-h APF wings (Fig. 3, A and A'). Loss of staining in clones expressing gishIR confirmed antibody specificity (Fig. 3, A and A'). A similar pattern was detected upon expression of Myc-Gish$^{\text{WT}}$ (Fig. 3, C and C'). Myc-Gish$^{\text{KD}}$ did not rescue the gishIR phenotype (see previous section) but was enriched at the membrane (unpublished data). Lastly, Myc-Gish$^{\text{C}}$, which did not rescue gishIR (see previous section), was ubiquitously localized in cells and prehairs (Fig. 3, D and D'). Analysis of subapical confocal projections (basal to prehair) revealed puncta and membrane

**Figure 3.** CK1-γ/Gish is associated with the base of the developing trichome. (A and A') 32–34-h APF wing actin-Gal4 FLP-out clones expressing UASgish$^{\text{IR}}$ (blue; yellow line marks clone border). Endogenous Gish (green and monochrome in A and A') is associated with the prehair base (prehair labeled with rhodamine-phalloidin, red). (B and B') Magnified view of A and A', outside UASgish$^{\text{IR}}$ expression clone. (C-D') 32–34-h APF pupal wings expressing UAS-myc-gish$^{\text{WT}}$ or UAS-myc-gish$^{\text{KD}}$ in actin-Gal4 FLP-out clones labeled with anti-Myc (green) and rhodamine-phalloidin (red). (C and C') Gish is observed upon expression of Myc-Gish$^{\text{WT}}$ (green or monochrome in C or C'). (D and D') C-terminal truncation, Myc-Gish$^{\text{AC}}$, is ubiquitously localized (green or monochrome in D or D'). (E and F) Basal projections of punctate and membrane localization of endogenous Gish (E) or Myc-Gish$^{\text{WT}}$ (F) proteins. Bars: [A] 10 µm; [B-F] 5 µm.
association for both endogenous and Myc-GishWT proteins (Fig. 3, E and F). In summary, CK1-γ/Gish is associated with the cell membrane and base of elongating prehairs.

**CK1-γ/gish regulates trichome morphogenesis in parallel to the PCP pathway**

Fz promotes trichome formation, and increased Fz levels result in multiple trichomes, likely through ectopic activation of the nucleation machinery (Krasnow and Adler, 1994). We reasoned that if the gishR phenotype resulted from Fz misregulation, removal of fz (null fz23 allele) would reduce the gishR effect. We observed no change in the gish phenotype in the fz-null background (Fig. S3 D), suggesting that gish function is Fz independent. Furthermore, as gish LOF does not include trichome orientation defects, a characteristic of core PCP phenotypes, we hypothesized that gish may be linked to an effector cascade downstream of the core PCP group. We thus examined potential genetic relationships between gish and known effectors of the core PCP factors that control trichome number.

The Drokzipper (zip) arm of PCP effector signaling failed to interact genetically with CK1-γ/gish (unpublished data). Furthermore, Zip localization or activation was not affected in gishR tissue (Fig. S3, E–F). We next analyzed the FH3 domain protein mwh, which is a PCP effector required to restrict actin filament formation and trichome number at the apical cell cortex (Wong and Adler, 1993; Strutt and Warrington, 2008; Yan et al., 2008). The null allele mwh1 dominantly enhanced the phenotype of gishGISh homozygous mutant clones (Fig. 4, A and B). Coexpression of mwhR and gishR also resulted in a synergistic enhancement of trichome number as compared with expression of either RNAi alone (unpublished data). We expressed gishR in an mwh-null mutant background, reasoning that modification of the null phenotype would support a parallel relationship. Strikingly, expression of gishR strongly enhanced the mwh-null phenotype (Fig. 4, D–F; quantification). These data support a genetic model whereby CK1-γ/gish and mwh cooperate in a parallel manner to restrict trichome formation.

As CK1-γ/gish and mwh appeared to cooperate to restrict trichome formation in parallel, we wished to determine whether overexpression of one could suppress the defect of the other. Expression of either Mwh or Myc-GishWT alone had no phenotype (not depicted), whereas expression of Mwh strongly suppressed the gishR phenotype (Fig. 4, G, H, and M, quantification). Similarly, overexpression of Myc-GishWT suppressed the mwhR phenotype (Fig. 4, I, J, and M, quantification), whereas Myc-GishKD failed to do so (Fig. 4, K and M, quantification). Lastly, the ubiquitously localized Myc-GishWT (see previous section) also failed to suppress mwhR (Fig. 4, L and M, quantification). Collectively, these data suggest that mwh and CK1-γ/gish (via kinase activity and membrane localization) cooperate in a parallel to regulate trichome formation.

**CK1-γ/gish regulates Rab11-mediated trafficking**

The polarized localization of yeast CK1-γ to regions of membrane deposition/bud growth (Robinson et al., 1999), the morphogenesis phenotype of YCK1/YCK2 (Robinson et al., 1993), and the sperm individualization phenotype of Drosophila CK1-γ/gish (Nerusheva et al., 2009) support a role for CK1-γ in membrane dynamics (see Introduction). We hypothesized that CK1-γ/gish function in trichome morphogenesis was related to polarized membrane trafficking.

To test this hypothesis, we analyzed well-characterized markers, including the recycling endosome-associated Rab11 GTPase (Ullrich et al., 1996; Dollar et al., 2002; Pelissier et al., 2003). During early prehair formation (30–32 h APF), Rab11 was detected in puncta in the subapical region (Fig. 5 A’’ and Fig. S4 B, diagram) and was detected in strongly stained foci at the base of the initiating prehairs in the apical region (Fig. 5, A and A’, yellow arrowheads; and Fig. S4 B). Strikingly, the apical Rab11 foci were derived from the prehair base of gishR-expressing cells, leaving mostly diffuse apical puncta (Fig. 5, A and A’). Abnormal Rab11 localization was also apparent in gishR clone tissue in 32–34 h APF wings; Rab11 was present uniformly in subapical cell regions in wild-type and gishR cells (Fig. 6, B’’ and D–D’, z sections), but puncta detected along the length of the developing prehairs in wild-type cells (Fig. 5, B and B’, insets) were mostly absent in gishR cells (Fig. 5, B, B’, and E, quantification). Again, diffuse apical Rab11 staining remained in the gishR tissue (Fig. 5 B’). In support of these data, the Rab11 binding partner Drip1 (Drosophila class 1 Fip; Prekeris et al., 2000; Li et al., 2007) was enriched to the prehairs but appeared mislocalized in gishR tissue (Fig. S4, G and G’). The Rab11-interacting protein MyoV (Hales et al., 2002) did not localize to the prehairs (Fig. S4, F and F’), suggesting a context-specific association of these proteins. Furthermore, Rab11 localization is likely dependent on Gish kinase activity and membrane enrichment, as we detected a partial rescue of Rab11 localization only upon coexpression of the GishWT transgene in gishR tissue (Fig. S1, I–L’). These data suggested that CK1-γ/gish was required to enrich Rab11 endosomal structures to the developing prehairs.

As CK1-γ/gish and mwh interacted in trichome restriction, we tested whether mwh regulated Rab11 localization in a manner similar to CK1-γ/gish. mwhR-expressing cells exhibited normal Rab11 localization to elongating prehairs (Fig. 5, C, C’, and E, quantification). These data and the genetic analysis led us to conclude that the functions of CK1-γ/gish and mwh converge to regulate trichome morphogenesis but use distinct cellular mechanisms (see Discussion).

To confirm Rab11 localization and to visualize recycling endosome and vesicle dynamics near the prehairs, we performed in vivo imaging of YFP-Rab11WT trafficking in the dorsal thorax (notum). At 36 h APF, we observed dynamic YFP-Rab11WT foci enriched to the base and within the developing prehairs, as revealed by the membrane-associated CD8-RFP (Fig. 5 F and Video 1). In contrast, coexpression of gishR exhibited a diffuse YFP-Rab11 pattern (Fig. 5 G and Video 2), similar to endogenous Rab11. At 38 h APF, we observed trafficking of YFP-Rab11WT within the elongating prehair (Fig. 5, H–J, yellow arrowheads; and Video 3). This activity was markedly reduced in the presence of a gishR trichome phenotype (Fig. 5, K–M, magenta arrowhead indicates remaining trafficking; and Video 4). These data support a requirement for gish in Rab11 dynamics associated with developing prehairs.
We extended the endosomal localization experiments to other markers of vesicle trafficking. In gish\textsuperscript{IR}-expressing cells, we found no defects in the level or localization of Rab5, which is required for early endocytic trafficking (Fig. S4, A–A'); Bucci et al., 1992), the endosomal marker Hrs (Fig. S4, C–C'); Lloyd et al., 2002), or the Golgi marker Lava Lamp (Fig. S4, D–D'; Sisson et al., 2000). As studies in yeast have supported a role for CK1-\(\gamma\) in endocytosis (Panek et al., 1997; Marchal et al., 2000), we analyzed dextran uptake in third-instar wing disc FLP-out clones expressing gish\textsuperscript{IR} but observed no effects...
Figure 5. CK1γ/gish regulates dynamic apical Rab11 trafficking. (A and A') Endogenous Rab11 in apical confocal projections of 30–32 h APF pupal wings (Fig. S4 B, diagram). actin-Gal4 FLP-out clones expressing UAS-gishIR (blue) show an absence of Rab11 foci (green and monochrome in A and A') at the trichome base (rhodamine-phalloidin, red; yellow arrowheads indicate Rab11 foci at the base of initiating prehairs). (A'') Rab11 in subapical projections of the same gishIR clones from A and A'. (B–B'') In 32–34 h APF wings, Rab11 puncta (see insets) were absent within trichomes in UAS-gishIR clones (blue; B and B''), whereas subapical regions appeared normal (B''). (C and C') actin-Gal4 FLP-out clones of UAS-mwhIR (blue) exhibit no effect on Rab11 localization (green and monochrome in C and C'). (D–D'') Z sections of wings from B–B'' (yellow lines indicate the gishIR clone border, which is blue in D). (E) Quantification of the percentage of cells with Rab11 puncta in multiple and single gishIR mutant trichomes and multiple mwhIR trichomes. (right) Rab11 detection in wild-type (nonclone [nc]) tissue is included to assess antibody/assay sensitivity. Error bars indicate SDs; unpaired t tests were performed on three independent animals (***, P < 0.001). (F–M) Time-lapse images of live notum epithelia highlighted with pnr-Gal4 expressed UAS-mCD8-RFP to mark membranes and UAS-YFP-Rab11WT labeling recycling endosomal structures. All images show nota oriented with anterior to the left.
To further dissect the gish and Rab11 CA interaction, we analyzed YFP-Rab11CA localization in 32–34-h APF wing cells. Expression of gishIR resulted in diffuse YFP-Rab11CA localization (Fig. S5, B and B'), in contrast to focused Rab11CA associated with control prehairs (Fig. S5, A and A'). These data indicated that the distribution of endogenous Rab11, YFP-Rab11 WT, and constitutively active YFP-Rab11CA is altered in gishIR cells. The enhancement of the Rab11CA effect by gishIR correlates with the diffuse localization of Rab11 CA away from the trichome. Furthermore, to determine the localization of Rab11 on an ultrastructural level, we analyzed the distribution of YFP-Rab11 in pupal wings by immuno-EM. In the pupal wing, YFP-Rab11 was observed in trichomes near the membrane (Fig. S5, E–E''), black arrows), and abundant YFP-Rab11–positive areas were identified in the apical cell region (Fig. S5 F).

Rab11 and effectors are required for trichome formation

The model that gish enriches Rab11 vesicles to promote single trichomes suggests vesicle recycling is required to deliver trichome nucleation activity. Therefore, we analyzed the effect of decreasing Rab11 function. Expression of a dominant-negative Rab11,

Figure 6. CK1-γ/gish and Rab11 genetically interact during trichome formation. (A) Strong expression of constitutively activated en-Gal4 UAS-YFP-Rab11 CA (29°C) resulted in multiple trichomes in adult wings. (B and C) UAS-YFP-Rab11 CA expression at 25°C via en-Gal4 exhibited no phenotype (B), whereas a single allele of gishIR induced multiple trichomes (C). (D and E) The en-Gal4 UAS-gishIR phenotype was synergistically enhanced by co-expression of UAS-GFP-Rab11 CA at 25°C. Distal is to the right in the images. (F) The quantification of results from B–E is represented. Error bars indicate SDs; unpaired t test was performed on three independent animals (*, P < 0.05). Bars, 25 μm.

(F) 36-h APF notum displays YFP-Rab11 WT localization to the base and within small prehairs (red). (G) The YFP-Rab11 WT pattern is lost upon UAS-gishIR coexpression. (H) 38-h APF notum shows YFP-Rab11 WT (green) localization along the length of the prehair (red). ([H–J] Time-lapse images of YFP-Rab11 WT dynamics (yellow arrowheads mark initial position shown in H' and serve as a reference in I and J). (K) 38-h APF notum coexpressing UAS-gishIR displays diffuse YFP-Rab11 WT localization and rare trafficking in prehair [red]. ([K–M] Time-lapse images tracking remaining YFP-Rab11 WT dynamics in prehair (magenta arrowheads mark trafficking shown in K' and serve as a reference in L and M); also see Videos 1, 2, 3, and 4. Yellow lines mark clone borders. Bars: (A–D) 10 μm; (F–H, and K) 5 μm.
proteins. The Rab11 effector nuf can bind Rab11 and link recycling endosome trafficking along microtubules via association with dynein (Riggs et al., 2007; Horgan et al., 2010). Nuf enrichment to the prehair base was dissociated in the apical domain of gishIR tissue (Fig. 7, B and B'). We next explored the morphological effect of reducing Nuf by analyzing nufIR in adult wings. nufIR expression resulted in shortened or malformed trichomes (Fig. 7, C [arrowheads and asterisks] and D [quantification]). We also observed patches of missing trichomes (unpublished data). In addition, the exocyst component Sec15 is a downstream effector required for polarized membrane delivery (Langevin et al., 2005; Wu et al., 2005; Oztan et al., 2007). The Rab11–Sec15 complex is required to initiate the formation of the exocyst and promote tethering of recycling endosome–derived vesicles to the plasma membrane for subsequent membrane fusion (Wu et al., 2008).
We therefore analyzed the effect of reducing Sec15 function on trichome development. To circumvent early lethality, we initiated expression of Sec15

induced defects in trichome development, similarly to Rab11 and Nuf, and, strikingly, caused an accumulation of Rab11 (see also Jafar-Nejad et al., 2005; Langevin et al., 2005) away from the base of the trichome across the cell (Fig. 7, compare E with F–F’). Accumulated Rab11 and defective trichome formation in the context of Sec15 knockdown, in conjunction with the Rab11 and Nuf phenotypes displaying missing and malformed prehairs, reveal (a) a requirement for Rab11–Nuf–Sec15 trafficking in trichome formation and (b) a requirement for the Sec15–exocyst complex in localized trafficking of nucleation activity through the Rab11 recycling endosome to nucleation sites.

Figure 8. CK1\(\gamma\)/gish regulates Rab11 effector Sec15GFP. (A–A’) Endogenous Sec15 (green and monochrome) is reduced in apical gish\(^{\text{IR}}\) tissue (blue; yellow lines mark clone border). (B–C’) nub-gal4 UAS-gish\(^{\text{IR}}\) disrupts Sec15GFP aggregates in 30-h APF pupal wings (C and C’) compared with nub-gal4 control tissue (B and B’; Fmi in magenta marks the membrane). (D and D’) The mwh\(^{1}\)-null allele has no effect on Sec15GFP aggregates. (E) Quantification of the mean number of Sec15GFP puncta per cell. Error bars indicate SDs; unpaired t tests were performed on three independent animals (**, P < 0.01). (F and F’) nub-Gal4 UAS-Sec15GFP expression in 30-h APF wings reveals large punctate colocalization of Sec15GFP and Rab11 (blue and monochrome in F and F’). (G and G’) nub-Gal4 coexpression of UAS-Sec15GFP and UAS-gish\(^{\text{IR}}\) reveals dissociation of both Sec15GFP and Rab11 (blue and monochrome in G and G’). Bars, 10 µm.

As sec15\(^{\text{IR}}\) exhibited effects on Rab11 localization and trichome formation, we analyzed the effect of gish\(^{\text{IR}}\) on endogenous Sec15. In 32–34-h APF wings, Sec15 was observed in a punctate distribution in the prehair, whereas apical Sec15 was mislocalized in gish\(^{\text{IR}}\) tissue (Fig. 8, A–A’). Furthermore, large apical patches of aggregated vesicles (dependent on exocyst function) have been reported in yeast, Drosophila epithelia, and mammalian cells upon Sec15GFP expression (Salminen and Novick, 1989; Zhang et al., 2004; Guichard et al., 2010). Thus, as an assay for Sec15GFP localization/function, we analyzed the formation of these large puncta in pupal wing cells (Fig. 8, B, B’, and E, quantification). Strikingly, in gish\(^{\text{IR}}\) cells, we observed an overall
necessary to restrict the nucleation machinery to a central subdomain (Adler, 2002). The function of CK1-γ/\(\gamma\)gish in polarized vesicle trafficking provides a mechanism for refining a trichome to a single position.

Gish is localized to the cell periphery and the prehair base. This localization is consistent with a requirement for CK1-γ/\(\gamma\)gish in targeting nucleation activity to that region, as we have identified ectopic trichome nucleation as the primary defect. Moreover, our data implicate vesicle trafficking, as genetic alteration of Rab11 or effectors (\(nuf\) and Sec15) resulted in lost and/or malformed trichomes. Collectively, these data support a model that targeted membrane recycling through Rab11, Nuf, and Sec15 is required to build a trichome (Fig. 9, compare A and B), and spatial regulation of this trafficking by CK1-γ/\(\gamma\)gish is required to counteract lateral membrane localization of nucleation activity and ectopic trichome formation (Fig. 9, A and C). A similar model exists for polarized growth in yeast (e.g., bud growth), in which uptake and recycling is a mechanism to balance cdc42 lateral diffusion (Marco et al., 2007; Slaughter et al., 2009).

CK1-γ/\(\gamma\)gish coordinates membrane recycling through Rab11–Nuf–Sec15 vesicle trafficking

Our data provide evidence that Rab11–Nuf–Sec15 trafficking promotes trichome formation. Importantly, this study supports that CK1-γ/Gish regulates localized actin nucleation by directing Rab11–Nuf–Sec15-mediated vesicle trafficking between the recycling endosome and a distinct region of the membrane. Evidence for polarized recycling includes that (a) Sec15\(^{IR}\) tissue displays trichome malformation in the presence of accumulated Rab11 vesicles away from the trichome base (reduced vesicle tethering and fusion can result in vesicle accumulation in the cell) and (b) dissociation of these large puncta (Fig. 8, C, C′, and E; and Fig. S4, H–I′), whereas mwh-null wings resembled the control (Fig. 8, D–E). Rab11 colocalized with Sec15GFP as previously reported (Zhang et al., 2004; Jafar-Nejad et al., 2005; Guichard et al., 2010), supporting that these structures were recycling vesicles trafficking to the plasma membrane (Fig. 8, F and F′). Sec15GFP and Rab11 were dissociated by gish\(^{IR}\) (Fig. 8, G and G′). Collectively, our data suggest that CK1-γ/\(\gamma\)gish specifically regulates Rab11–Nuf–Sec15 vesicle localization and polarized trafficking between the recycling endosome and the plasma membrane.

Discussion

CK1-γ/\(\gamma\)gish regulates membrane trafficking to coordinate cell and tissue morphogenesis

Membrane trafficking is a key mechanism during morphogenesis and cell polarization (Lecuit, 2003; Mellman and Nelson, 2008). Studies in Drosophila have established a requirement for trafficking in core PCP protein localization, trichome orientation, and morphogenesis (Shimada et al., 2006; Strutt and Strutt, 2008; Fricke et al., 2009; Mottola et al., 2010; Pataki et al., 2010; Purvanov et al., 2010). Membrane trafficking has also been associated with PCP establishment in cell packing (Classen et al., 2005) and in vertebrate morphogenesis during cilia formation (Park et al., 1994; Gray et al., 2009). In the Drosophila wing, the core PCP proteins are required for the formation of a single trichome at the distal cell vertex, but these proteins are found along the entire distal (Fz/Dsh) and proximal (Stbm–Van Gogh/Prickle) cell sides (Adler, 2002). It is unclear how trichomes are restricted to one position within a broader PCP domain. It is likely that proteins, such as Fz, broadly define prehair formation and, within that domain, recruit the refinement proteins necessary to restrict the nucleation machinery to a central subdomain (Adler, 2002). The function of CK1-γ/\(\gamma\)gish in polarized vesicle trafficking provides a mechanism for refining a trichome to a single position.

Gish is localized to the cell periphery and the prehair base. This localization is consistent with a requirement for CK1-γ/\(\gamma\)gish in targeting nucleation activity to that region, as we have identified ectopic trichome nucleation as the primary defect. Moreover, our data implicate vesicle trafficking, as genetic alteration of Rab11 or effectors (\(nuf\) and Sec15) resulted in lost and/or malformed trichomes. Collectively, these data support a model that targeted membrane recycling through Rab11, Nuf, and Sec15 is required to build a trichome (Fig. 9, compare A and B), and spatial regulation of this trafficking by CK1-γ/\(\gamma\)gish is required to counteract lateral membrane localization of nucleation activity and ectopic trichome formation (Fig. 9, A and C). A similar model exists for polarized growth in yeast (e.g., bud growth), in which uptake and recycling is a mechanism to balance cdc42 lateral diffusion (Marco et al., 2007; Slaughter et al., 2009).
Rab11–Nuf–Sec15 from the prehair in *gish*<sup>‡</sup> cells is associated with multiple trichomes. These results suggest that polarized and focused membrane delivery of a nucleation factors may be disrupted. Interestingly, knockdown of the Arp2/3 nucleation machinery or regulators, such as Wasp, resulted in multiple trichomes (Fig. S5, G–I; Fricke et al., 2009) and can genetically enhance the *gish*<sup>‡</sup> phenotype (Fig. S5, J–M, quantification). CK1-γ/Gish may promote delivery of Rab11–Nuf–Sec15 recycling vesicles carrying Arp2/3 activity to the prehair. Further experiments are needed to explore the relationship between localized Rab11 trafficking and the branched nucleation machinery.

As Gish is associated with the base of developing trichomes, it is possible that CK1-γ/gish, in analogy to the yeast homologue Yck2p, is localized to the proximal prehair membrane in an area of membrane deposition. Such localization suggests Gish could regulate localized trafficking by tethering Rab11 vesicles to the plasma membrane through the Sec15–exoyctosome complex. As in other contexts, Rab11 and Sec15 are functionally linked on vesicles en route from the recycling endosome to the plasma membrane. As *gish* reduction mislocalized endogenous Rab11–Nuf–Sec15 and dissociated the aggregated Sec15GFP and Rab11 colocalization pattern (but had no effect on alternative trafficking compartments, such as the Rab5 early endosome), this suggests CK1-γ/gish is required at a late vesicle recycling step. Although coimmunoprecipitation experiments with Myc-Gish failed to detect an association with Rab11, Nuf, or Sec15 (unpublished data), the potential phosphorylation targets may be binding partners or associate through weak/transient interactions with Gish.

Alternatively, CK1-γ/gish may regulate polarized vesicle trafficking through an effect on the cytoskeleton, associated motor proteins, or adaptors. As mentioned, the Rab11 effector Nuf can bind the motor protein dynein and link Rab11 endosomal structures to microtubules (Riggs et al., 2007; Horgan et al., 2010). A recent study showed that Nuf phosphorylation by IKK-ε regulates trafficking of Rab11 vesicles along developing bristles (Otani et al., 2011). Thus, one possibility is that CK1-γ/gish may act through Nuf to affect Rab11 trafficking. We have observed a correlation between Rab11 localization and microtubule networks within the prehair (unpublished data). Thus, Rab11 vesicles may require microtubules for localization, as previously reported in other contexts (Riggs et al., 2007). Consistent with this notion, microtubule depolymerization with nocodazole dissociated Sec15 vesicle aggregates in mammalian cells (Zhang et al., 2004). Intriguingly, disrupting microtubules resulted in multiple trichomes in *Drosophila* (Turner and Adler, 1998).

Further analysis is necessary to address the role of *gish* on microtubule-based vesicle trafficking in the context of trichome formation.

**CK1-γ/gish and mwh regulate distinct steps in trichome morphogenesis**

PCP studies have identified effectors required to restrict trichomes, such as _fuzzy_, _inturned_, _frtz_, and _mwh_ (Adler, 2002). Based on genetic epistasis and localization experiments, _mwh_ is downstream as a modulator of the cytoskeleton (Wong and Adler, 1993; Strutt and Warrington, 2008; Yan et al., 2008). Mwh is enriched proximally, and a model has been proposed whereby prehair initiation is restricted to the distal cell region by the proximal repression of Mwh (and promoted by Fz in the distal domain). Taking these observations together with our data regarding the relationship of _mwh_ and CK1-γ/gish, the restriction of prehair initiation requires two parallel mechanisms in orthogonal axes: (1) prehair initiation is restricted by the gradient of Mwh along the PD axis (e.g., wing) of the cell and (2) the tight restriction of Fz-directed trichome nucleation along a second axis of refinement by CK1-γ/gish (Fig. 9). This model is supported by both genetic and cell biological data: (a) analyses of CK1-γ/gish and _mwh_ LOF reveal phenotypic differences—the initial prehair phenotype of CK1-γ/gish is restricted to the distal cell region and results in multiple, distally oriented trichomes, whereas the _mwh_ phenotype is first observed as excess actin filaments over the entire apical cell cortex and results in multiple, randomly oriented trichomes (Strutt and Warrington, 2008), (b) _gish*<sup>‡</sup> strongly enhances the _mwh*-null phenotype, indicative of two genetically independent pathways, and (c) CK1-γ/gish is required for Rab11 localization in the proximity of the developing prehair, whereas _mwh_ has no effect on Rab11 (or Sec15GFP). Collectively, these data imply that these proteins perform two independent functions in the cell that converge to restrict trichome formation to a single site. In support of this model, our data indicate that excess of either of these proteins can partially suppress the defects associated with the loss of the other. Thus, we suspect a failure to focus trichome nucleation activity to the distal cell vertex by *gish* LOF can be corrected by increasing Mwh levels to repress actin nucleation/polymerization by an independent mechanism.

In summary, we define a novel mechanism by which trichome formation is restricted to a single domain in epithelial cells. These data support the model that CK1-γ kinases can regulate cellular morphogenesis through controlling the localization of Rab11 vesicle recycling. Our study has identified parallels between *Drosophila* and yeast CK1-γ in cellular morphogenesis, supporting a conserved mechanism of action. Collectively, our findings reveal that an independent mechanism of CK1-γ-regulated vesicle trafficking converges to refine Fz/PCP-directed morphogenesis.

**Materials and methods**

**Fly stocks**

*Drosophila* experiments were performed at 25°C unless otherwise indicated. Deficiency collection stocks used in the modifier screen were obtained from Exelixis, Szeged, and Bloomington Stock centers. Phenotypic analysis was performed on genes isolated in the modifier screen through transgenic UAS-RNAi flies obtained from the Vienna *Drosophila* RNAi Center (Dietzl et al., 2007). The following alleles were used in this study and obtained from the Bloomington Stock Center: Drok<sup>‡</sup>, zip<sup>‡</sup>, *gish*<sup>‡</sup>, *gish<sup>‡</sup>*<sup>zip1</sup>, _mwh<sup>‡</sup>_ and _scarb<sup>‡</sup>_ (described in Flybase). _wog<sup>‡</sup>_ was a gift from E.D. Schejter (Weizmann Institute of Science, Rehovot, Israel; Ben-Yaacov et al., 2001). *gish*<sup>‡</sup><sup>j8759</sup> was recombined onto an FRT82B chromosome, and mitotic clones of FRT82B-*gish*<sup>‡</sup><sup>j8759</sup> were generated via the FLP/FRT system (Xu and Rubin, 1993). Clones were unmarked or marked with the forked mutation in adults or marked by the absence of β-galactosidase in pupal tissue. *gish<sup>‡</sup>*<sup>zip1</sup>-GFP is a
protein trap insertion within the gish locus, generating a Gish-GFP fusion protein (Morin et al., 2001; Frescas et al., 2004). Overexpression of cDNA transgenes or RNAi (IR) was performed using the Gal4/UAS system (Brand and Perrimon, 1993). The Gal4 expression drivers used were as follows: sev-Gal4, en-Gal4, nub-Gal4, and dpp-Gal4. eFlp-out expression clones of the indicated restriction sites were generated using hs-FLP; actin-Gal4, UAS-GFP (Struhl and Basler, 1993), UAS-gish (v26003), UAS-mwh (v41198), UAS-Rab11IR (v21198), and UAS-SE15 (v105126), and UAS-PTEN (v35731) were obtained from the Vienna Drosophila RNAi Center collection. Where indicated, UAS-icer2 was included with UAS-gish expression to increase RNAi efficiency (Bloomington Stock Center). The following transgenes were generated from the Berkeley Drosophila Genome Project gish cDNA clone LD04357. UAS-gishP2 was created by PCR amplifying a cDNA sequence (independent of the Vienna Drosophila RNAi Center probe sequence; Fig. S1 A) into a modified pWizDir vector (entire sequence, GenBank accession No. EF096381). The PCR primers used to generate gishP2 were (including restriction sites) forward, 5′-ATCTCAGGTCTCCGCGATCGAATATTGTT-3′, and reverse, 5′-GCTGGATCCACGCGGATCCCTGTATCTC-3′. UAS-myc-gishP2 and UAS-myc-gish were PCR isolated and correspond to transcripts gish-RB and gish-RF, respectively (described in Flybase). The PCR primers used to generate these transcripts were (including restriction sites and linkers) forward primer (common for gish-RB and gish-RF), 5′-ATCG-GGATCCCGCGGATCCCTGTATCTC-3′, and reverse primer, 5′-GCTGGATCCACGCGGATCCCTGTATCTC-3′, and reverse gish-RF only, 5′-GCTCTCGAGATCCGATCCGATCCGCTTGCATTTG-3′, and reverse gish-RF only, 5′-GCTCTCGAGATCCGATCCGATCCGCTTGCATTTG-3′. These PCR products were cloned into pCS2-Myc, and myc-gish was subcloned into the pUAST vector to generate transgenic flies. myc-gishP2 (D187N substitution) was generated by site-directed mutagenesis (Agilent Technologies) of pCS2-myc-gishP2 followed by subcloning of myc-gishP2 into pUAST. The PCR primers used to generate the D187N substitution were forward, 5′-GCCACTAATATAATGGAATGGAACACCGAGAAAC-3′, and complementary reverse, 5′-GTCCTTGATCATTGGGTCCTGCATT-3′. The percent increase of the area of mutant tissue was calculated relative to adjacent control tissue in the same wing. Means and SDs were calculated from at least three wings from individual animals. Sec15GFP puncta number per cell was determined using the particle analysis function of ImageJ. Three independent wings were analyzed, and the number of Sec15GFP-positive cells in each cell was used to determine the mean number of puncta per cell.

Live imaging of pupal notum

White pupae (0 h APF) of the indicated genotypes were collected into separate vials, aged to 36 or 38 h APF at 25°C, and mounted for imaging as previously described (Bellaïche et al., 2001). In brief, aged pupae were fixed on slides with double-sided tape in between stacks of four coverslips. Pupal cases were partially removed to expose head and notum. A drop of halocarbon oil (Sigma-Aldrich) was placed onto the bottom of a cover slide and gently applied to the notum, supported by the adjacent stacks of coverslips. Images were acquired at room temperature using a confocal microscope (63 x oil immersion, 1.4 NA; SP5 DMI; Leica) with LAS AF (Leica) software. A single confocal plane (1 µm) was taken at 6-s intervals for a total of 2 min.

Dextran uptake assay

The dextran uptake assay was performed as previously described (Entchev et al., 2000) with some modifications: third-instar larvae were partially dissected in 52 medium supplemented with 10% FBS to expose wing discs. The wing disc was replaced with 5 µl of dextran (lysin fixable; 3,000 molecular weight; Invitrogen) and pulsed for 10 min at 25°C. The samples were washed three times with ice-cold S2 medium (with 10% FBS) and then incubated at 25°C for 20 min (chase) to visualize uptake and the early endosomal compartment. The samples were fixed for 20 min in 4% formaldehyde in PBS and washed three times in PBS with 0.1% Triton X-100. Mounting was in 30% glycerol (Mowiol and 2.5% Dabco). Images were acquired at room temperature using a confocal microscope (63 x, 1.4 NA; LSM 510 Meta) using LSM software. Images were processed with ImageJ and Photoshop CS4.

EM

White pupae (0 h APF) of the indicated genotypes were isolated and staged to 32 h APF, and pupal wings were dissected as described in Immunohistochemistry and histology. Wings were fixed in 3% glutaraldehyde and then in 1% osmium tetroxide. Samples were dehydrated in a graded ethanol series (50, 70, 90, and 100%) and propylene oxide and embedded in Durcupan resin. Tanganental sections were made and mounted on a slide using DPX mounting medium. Adult wings, notae, and eye sections were imaged at room temperature on a microscope (Axioiapan; Carl Zeiss). Images were acquired with a camera (Zeiss AxioCam Color type 412–312; Carl Zeiss) and AxioCam software. For analysis of pupal wings, white pupae were collected (0 h APF) and aged at 25°C. Dissections were performed as follows: in brief, pupae were immobilized on double-sided tape, removed from the pupal case, and placed into PBS, in which pupae were partially dissected to remove fat tissue and then fixed in 4% formaldehyde in PBS and washed three times (PBS and 0.1% Triton X-100), and then placed in 80% glycerol in PBS. Notae were then fully dissected and mounted on a slide in 80% glycerol in PBS. Adult eye section analyses were performed as previously described (Gaengel and Mlodzik, 2008). In brief, fly heads were fixed in 2% glutaraldehyde in PBS and treated with 2% osmium tetraoxide. Samples were dehydrated in a graded ethanol series (50, 70, 90, and 100%) and propylene oxide and embedded in Durcupan resin. Tangential sections were made and mounted on a slide using DPX mounting medium. Adult wings, notae, and eye sections were imaged at room temperature on a microscope (Axioiapan; Carl Zeiss). Images were acquired with a camera (Zeiss AxioCam Color type 412–312; Carl Zeiss) and AxioCam software. For analysis of pupal wings, white pupae were collected (0 h APF) and aged at 25°C. Dissections were performed as follows: in brief, pupae were immobilized on double-sided tape, removed from the pupal case, and placed into PBS, in which pupae were partially dissected to remove fat tissue and then fixed in 4% formaldehyde in PBS and washed three times (PBS and 0.1% Triton X-100). Wing membranes were removed, and samples were incubated in wash buffer containing 10% normal goat serum overnight for primary antibody (4°C), washed three times with PBS, and then incubated with secondary for 1 h (25°C). Wings were washed three times with PBS and mounted in glycerol/PBS supplemented with 1% N-propyl gallate. Pupal wing images were acquired at room temperature using a confocal microscope (63 x, 1.4 NA; LSM 510 Meta; Carl Zeiss) using LSM software (Carl Zeiss). Images were processed with ImageJ (National Institutes of Health) and Photoshop CS4 (Adobe).
at 4°C. Papae were washed five times in glycerol-PBS and then incubated for 3 h with secondary antibody and washed five times. All subsequent steps were as described in the previous paragraph for standard TEM. The electron microscope used in all cases was an H-7650 (Hitachi) with Maxim DL software (Diffraction Ltd.).

Immunoblotting

Third-instar wing imaginal discs (50 discs/genotype) were dissected and placed directly into SDS sample buffer to dissolve the tissue. These samples were boiled at 95°C for 10 min and then centrifuged at 14,000 rpm for 10 min. The supernatant was run on a 10% SDS-PAGE gel and transferred to polyvinylidene fluoride membrane (Millipore). The membranes were probed with rabbit antiphospho-MRLC (Ser19; Cell Signaling Technology). ECL Plus was used for detection (GE Healthcare).

Cell culture and siRNA

293T cells were cultured in DME supplemented with 10% FBS and maintained in 5% CO2 at 37°C. Cells were transfected with siRNA against human CSNK1G1, CSNK1G2, and CSNK1G3 or negative control siRNA (Silencer Select siRNA and negative control #1; Invivogen). Transfection was performed using siPORT NeoFX (Invivogen). After 48 h, cells were transfected with pEGFP-C3-GFP (0.4 µg per well; 8-well Laboratory-Tek glass insert slide; cell from C.A. Mitchell, Monash University, Clayton, Victoria, Australia; Zhang et al., 2004) with the transfection reagent (FuGENE HD; Promega). After 24 h, cells were fixed with 4% paraformaldehyde, washed in PBS (0.1% Triton X-100), and mounted with Vectashield (including DAPI; Vector Laboratories). Images were acquired using a confocal microscope (63x, 1.4 NA; LSM 510 Meta) using LSM software. Images were processed with ImageJ and Photoshop CS4.

Online supplemental material

Fig. S1 shows in vivo RNAi strategy used for gish knockdown and the requirement for CK1γ/gish kinase activity and membrane association in trichome formation and Rab11 localization. Fig. S2 displays data characterizing the apical membrane expansion phenotype of gish. Fig. S3 displays data supporting a PCP-independent role for CK1-γ/gish regulation of trichome morphogenesis. Video 1 shows YFP-Rab11 WT trafficking within initiating prehair in a 36-h APF notum. Video 2 shows diffuse localization of YFP-Rab11 WT with Rab11 WT trafficking within elongating prehair in a 38-h APF notum. Video 3 displays data showing that Rab11 WT trafficking with Rab11 WT trafficking within initiating prehair in a 36-h APF notum. Video 4 shows YFP-Rab11 WT trafficking with Rab11 WT trafficking within elongating prehair in a 38-h APF notum. Video 5 shows diffuse localization of YFP-Rab11 WT with Rab11 WT trafficking within elongating prehair in a 38-h APF notum. Video 6 shows Rab11 WT trafficking with Rab11 WT trafficking within elongating prehair in a 38-h APF notum. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201107137/DC1.

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Clifford, J., B. Robertson, T. May, M. Mlodzik, S. Zhang, and V. Penman. 2004. Diego interacts on YFP-Rab11 CA localization, YFP-Rab11 WT localization in coexpression during prehair elongating in a 38-h APF notum. Fig. S3 displays data supporting a PCP-independent role for CK1-γ/gish regulation of trichome morphogenesis. Video 1 shows YFP-Rab11 WT trafficking within initiating prehair in a 36-h APF notum. Video 2 shows diffuse localization of YFP-Rab11 WT with Rab11 WT trafficking within elongating prehair in a 38-h APF notum. Video 3 shows YFP-Rab11 WT trafficking with Rab11 WT trafficking within elongating prehair in a 38-h APF notum. Video 4 shows diffuse YFP-Rab11 WT trafficking with gish coexpression during prehair elongating in a 38-h APF notum. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201107137/DC1.

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Figure S1. gish requires kinase function and membrane localization for morphogenesis. (A) Diagram representing RNAi target sequence location for two independent RNAi lines as well as the GishWT and GishKC structure. The gish kinase-dead (KD) D187N mutation is based on the comparable Xenopus CK1-γ mutant on a conserved residue in the ATPase domain (Davidson et al., 2005). (B) en-Gal4 expression of a Vienna Drosophila RNAi Center-independent RNAi, gishIR2, generates multiple trichomes similar to gish and FRT82-gish101722. Distal is to the right. (C–C′) en-Gal4 UAS-gishWT eliminates gishpterGFP/+ (green) expression in the posterior en domain [red]. (D and E) UAS-myc-gishWT transgene coexpression partially rescues the en-Gal4 UAS-gishWT phenotype. (F and G) Two independent UAS-myc-gishWT transgenes (F and not depicted) and a ubiquitously localized UAS-myc-gishKC transgene (G) failed to rescue the phenotype. (H) Trichome quantification is graphically represented; the asterisk indicates a statistically significant rescue. Error bars show SDs (*, P < 0.05). (I–L) Rab11 localization is shown for the corresponding rescue experiments in D–G (yellow arrowheads indicate Rab11 association with the prehairs). Bars: (B and D–G) 25 µm; (C–C′) 20 µm; (I–L) 10 µm.
Figure S2. *gish* regulates apical membrane growth. (A–B’) Z sections of ~28-h APF pupal (29°C) *actin-Gal4* FLP-out clones (GFP) during prehair formation stained with rhodamine-phalloidin (red). (A and A’) Control FLP-out clones (GFP). (B and B’) *UAS-gish* expression induces apical membrane expansion. (C) The degree (percentage) of membrane expansion was measured within the xy plane (apical area was measured with Fmi protein, and basolateral area was measured with cortical rhodamine-phalloidin staining below the nuclear level, graphically represented as the increase in cell area relative to adjacent control tissue; *, P < 0.05; ***, P < 0.001; unpaired t tests on pupal wings of three independent animals were scored). (D and E) TEM images display excess cellular projections in *nub-Gal4* UAS-*gishIR* tissue at 32–34 h APF (E) in comparison with *nub-Gal4* control tissue (D). Red boxes indicate comparable regions between the images. (F and F’) Confocal projections of whole nuclei staining (Hoechst, blue and monochrome in F and F’) of ~28-h APF pupal wing (29°C) *actin-Gal4* FLP-out clones expressing UAS-*gishIR* (GFP; clone border marked with yellow line) reveal no change in area. (G) Nuclear area was measured and graphically represented. (H–H’’) Approximately 28-h APF (29°C) *actin-Gal4* FLP-out clones (GFP; clone border marked with yellow lines) expressing UAS-*PTENIR* reveal no trichome defects associated with increase cell size (rhodamine-phalloidin, red; Fmi, blue in H and H’ and monochrome in H’’). Error bars show SDs. Bars: [A, B, and F] 10 µm; [D and E] 2 µm; [H] 20 µm.
Figure S3. \textit{gish} function is independent of the Fz/Drok arm of the PCP pathway. (A–A') FLP/FRT \textit{gish}\textsuperscript{IR} mutant clone (absence of β-galactosidase [B-Gal], blue; clone border marked with yellow line in A') displaying multiple trichomes (red and monochrome in A and A'; yellow arrowheads) and asymmetric Fmi localization (green and monochrome in A). (B–C') 32–34 h APF pupal wings displaying Fmi (green and monochrome) and rhodamine-phalloidin (red). \textit{en-Gal4 UAS-gish}\textsuperscript{IR} expression does not affect asymmetric localization of Fmi (C and C') compared with a control wing (B and B'). (D) The \\textit{nuB-Gal4 UAS-gish}\textsuperscript{IR} trichome phenotype is not suppressed in an \textit{fz}\textsuperscript{P21}/\textit{fz}\textsuperscript{P21} (\textit{fz} null) background (wings of three independent animals were scored). Drok (Rho-associated kinase) restricts trichome number downstream of PCP proteins Dsh and RhoA and functions to activate Zipper (Zip; the \textit{Drosophila} myosin II homologue; Winter et al., 2001). We tested this Drok/Zip arm of PCP effector signaling by analyzing a dominant genetic interaction with the alleles \textit{zip}\textsuperscript{1}, Drok\textsuperscript{2}, or a constitutively activated \textit{spaghetti squash} (the \textit{Drosophila} homologue of MRLC, \textit{sqh}\textsuperscript{E20E21}; Winter et al., 2001) in the context of \textit{gish}\textsuperscript{IR}. In any case, we failed to detect a significant modification of the \textit{gish}\textsuperscript{IR} phenotype (not depicted). Error bars show SDs. (E–E') Zip staining (green in E and E' and monochrome in E') demonstrates no change in levels or localization in \textit{actin-Gal4 FLP-out UAS-gish}\textsuperscript{IR} clones compared with adjacent tissue (blue; clone border marked with yellow lines). Note that Zip staining can be observed within developing prehairs in control and multiple trichome \textit{gish}\textsuperscript{IR} tissue (rhodamine-phalloidin staining in red). (F) In third-instar wing discs expressing \textit{UAS-gish}\textsuperscript{IR} via \textit{en-Gal4} (29°C), no change in Sqh phosphorylation and thus Zip activation was observed in immunoblotting using a phosphospecific antibody for activated MRLC phosphorylated on a conserved serine (Ser21 in \textit{Drosophila} corresponds to mammalian Ser19; Matsumura et al., 1998; Winter et al., 2001). Bars, 10 µm.
**Figure S4.**  
_**gish** function is specific to Rab11 trafficking._ (A–D”) Examination of apical or subapical staining (see diagram in B) for Rab5 (green in A and monochrome in A’ and A”), Hrs (green in C and monochrome in C’ and C”), or Lava Lamp (Lva; green in D and monochrome in D’ and D”) in actin-Gal4 FLP-out UAS-gish clones (blue) revealed no difference in staining when compared with adjacent wild-type tissue. Distal is to the right. (B) Diagram defining the location of apical and subapical confocal projections in our assay (each bracket shows 1.2-µm projections). (E and E’) Dextran uptake (red and monochrome in E and E’) in third-instar wing disc actin-Gal4 FLP-out UAS-gish clones (green), TR, Texas red. (F–G’) 32–32h APF wings expressing actin-Gal4 FLP-out UAS-gish clones (blue) display normal MyoV and mislocalized dRip11 localization (green and monochrome in F and G and F’ and G’ respectively; yellow arrowheads indicate dRip11 enriched at the base of the prehair). (H and I) 293T cells transfected with Sec15GFP (gift from C. Mitchell) and control or siRNA targeting the three CSNK1G (mammalian CK1-γ) genes. Clone borders are marked with yellow lines. Bars: (A, C, D, F, and G) 10 µm; (E, H, and I) 20 µm.
Figure S5. gish, Rab11, and branched nucleators in trichome formation. (A–B′) 32–34-h APF pupal wings expressing nub-Gal4 UAS-YFP-Rab11CA. YFP-Rab11CA was shown associated with the elongating trichome (red), whereas coexpression of UAS-gishIR displayed a more diffuse YFP-Rab11CA localization (B and B′). (C and C′) Rab11IR Flip-out clones (blue and outlined in yellow) displayed reduced anti-Rab11 staining associated with the trichome, supporting that this localization pattern is specific to the endogenous Rab11 protein. (D and D′) Rab11IR actin FLP-out clones displaying delayed and malformed trichomes (red). Cell membranes are labeled with Fmi (blue). (E′) Enlarged view present in the yellow area in E. Black arrows indicate 10-nm gold particles labeling GFP-Rab11. Note the association of GFP-Rab11 on the trichome membrane; distal is up, and apical is to the right. (F) Apical tangential section displaying Rab11 localization (black arrows; distal is up). Note the adherent junctions (yellow arrows in E, E′, and F). (G–I) nub-Gal4 expression of Arp3IR and WaspIR and overexpression of a myristoylated form of the Wasp regulator Kette induce multiple trichomes (see also Fricke et al., 2009). (J–M) Removal of a single copy of wasp or scar/wave enhances the gishIR phenotype (quantification in M). Unpaired t test was performed on three animals. Error bars show SDs (*, P < 0.05; ***, P < 0.001). Bars: (A–D) 10 µm; (E) 1 µm; (E′ and F) 0.5 µm; (G–L) 25 µm.
Video 1. **Rab11 vesicle dynamics during trichome initiation.** 36-h APF notum expressing YFP-Rab11\(^{WT}\) (green) in association with initiating prehair in the \(pnr\) domain (plasma membrane highlighted with mCD8-RFP, red). Images were acquired by time-lapse confocal microscopy using a confocal microscope (63x, 1.4 NA; SP5 DMI) and Leica software. Single confocal planes (1 µm) were taken at 6-s intervals for a total of 2 min.

Video 2. **Rab11 vesicle dynamics are disrupted by gish\(^{IR}\) during trichome initiation.** 36-h APF notum expressing gish\(^{IR}\) and YFP-Rab11\(^{WT}\) (green) in the context of an initiating prehair in the \(pnr\) domain (plasma membrane highlighted with mCD8-RFP, red). Images were acquired by time-lapse confocal microscopy using a confocal microscope (63x, 1.4 NA; SP5 DMI) and Leica software. Single confocal planes (1 µm) were taken at 6-s intervals for a total of 2 min.

Video 3. **Rab11 vesicle dynamics during trichome elongation.** 38-h APF notum expressing YFP-Rab11\(^{WT}\) (green) in association with elongating prehairs in the \(pnr\) domain (white arrows; plasma membrane highlighted with mCD8-RFP, red). Images were acquired by time-lapse confocal microscopy using a confocal microscope (63x, 1.4 NA; SP5 DMI) and Leica software. Single confocal planes (1 µm) were taken at 6-s intervals for a total of 2 min.

Video 4. **Rab11 vesicle dynamics are disrupted by gish\(^{IR}\) during trichome elongation.** 38-h APF notum expressing gish\(^{IR}\) and YFP-Rab11\(^{WT}\) (green) in the context of an elongating prehair in the \(pnr\) domain (white arrows; plasma membrane highlighted with mCD8-RFP, red). Images were acquired by time-lapse confocal microscopy using a confocal microscope (63x, 1.4 NA; SP5 DMI) with Leica software. Single confocal planes (1 µm) were taken at 6-s intervals for a total of 2 min.

**References**


