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Dusky-like functions as a Rab11 effector for the deposition of cuticle during *Drosophila* bristle development

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SUMMARY

The morphogenesis of *Drosophila* sensory bristles is dependent on the function of their actin and microtubule cytoskeleton. Actin filaments are important for bristle shape and elongation, while microtubules are thought to mediate protein and membrane trafficking to promote growth. We have identified an essential role for the bristle cuticle in the maintenance of bristle structure and shape at late stages of bristle development. We show that the small GTPase Rab11 mediates the organized deposition of chitin, a major cuticle component in bristles, and disrupting *Rab11* function leads to phenotypes that result from bristle collapse rather than a failure to elongate. We further establish that Rab11 is required for the plasma membrane localization of the ZP domain-containing Dusky-like (Dyl) protein and that Dyl is also required for cuticle formation in bristles. Our data argue that Dyl functions as a Rab11 effector for mediating the attachment of the bristle cell membrane to chitin to establish a stable cuticle. Our studies also implicate the exocyst as a Rab11 effector in this process and that Rab11 trafficking along the bristle shaft is mediated by microtubules.

KEY WORDS: *Drosophila*, Rab11, Dyl, Microtubules, Bristle, Chitin, Cuticle, Growth, Transport, p150^{glued}

INTRODUCTION

Polarized extensions of epidermal cells, such as the sensory bristles, arista laterals and wing hairs of *Drosophila melanogaster*, are good model cell types for studying the processes that govern cytoskeleton-mediated morphogenesis (Fei et al., 2002; Guild et al., 2005; Tilney et al., 2004; Tilney et al., 1995; Tilney and DeRosier, 2005; Turner and Adler, 1998). However, little is known about the mechanisms that mediate membrane and protein transport during the growth of these structures. Here we report that decreased function in components of the intracellular trafficking and secretion machinery, such as the Rab11 GTPase, produces a dramatic mutant phenotype by a novel mechanism, namely bristle collapse, and that this is associated with a defect in cuticle deposition.

Time-lapse studies have shown that, except for the initial stages of bristle development when growth is primarily isotropic, bristle growth is highly polarized in the axial direction (Fei et al., 2002). A variety of genetic and inhibitor studies argue that actin and microtubules play different roles in bristle growth (Cant et al., 1994; Fei et al., 2002; He and Adler, 2001; Petersen et al., 1994; Tilney et al., 2000a; Turner and Adler, 1998). Large bundles of F-actin help to maintain the shape of the bristle, which is long and tapered with a small but reproducible curve, and the polymerization of actin at the tip has been thought to drive extension (Tilney et al., 1996; Tilney et al., 2000b). The microtubule cytoskeleton has been suggested to function in the transport of material along the proximal distal axis of the bristle (Fei et al., 2002; Guild et al., 2002; Tilney et al., 2000a). Once the bristle has reached its full length the actin filament bundles disappear and bristle shape is thought to be maintained by the chitinous exoskeleton (Tilney et al., 1996).

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Rab11 plays an important role in polarized transport in epithelial cells (Wang et al., 2000), in protein recycling from endosomes to the plasma membrane and in the trans-Golgi network (Band et al., 2002; Hales et al., 2001; Schlierf et al., 2000; Ullrich et al., 1996; Volpicelli et al., 2002; Wilcke et al., 2000). *Drosophila Rab11* mediates the transport of rhodopsin to the apical plasma membrane domain of growing rhabdomeres (Satoh et al., 2005), is required for membrane recycling during cellularization (Pelissier et al., 2003), organizes microtubule polarity in oocytes (Dollar et al., 2002) and is required for the asymmetric cell divisions that give rise to the cells of the bristle sensory organ (Emery et al., 2005). A role in bristle morphogenesis is implied by the bristle morphology phenotypes of hypomorphic *Rab11* genotypes (Jankovics et al., 2001).

We have examined the role of Rab11 in bristle morphogenesis, making use of the controlled expression of *Rab11* double-stranded (ds) RNA to knock down *Rab11* function after the formation of the sense organ lineage. This treatment led to the macrochaetae being reduced to short stubs. Surprisingly, in vivo imaging revealed that this extreme phenotype did not result from a failure in bristle elongation but was due to a failure in stability, as bristles expressing *Rab11* dsRNA grew and then collapsed. This was correlated with defects in the organization of chitin bands in the bristle cuticle, suggesting an important role for Rab11 in the secretion and patterned deposition of chitin. Our studies further implicate Dusky-like (Dyl), a ZP domain-containing protein, as a Rab11 effector for chitin deposition. Previous studies found that *dyl* functions in the formation of embryonic denticles, likely by mediating the connection between the apical plasma membrane and the forming cuticle (Fernandes et al., 2010). We found that *dyl* was required for the organized deposition of chitin in bristles and further that Rab11 function was required for the plasma membrane localization of Dyl. The exocyst is known to be a Rab11 effector in many contexts (Emery et al., 2005; He and Guo, 2009; Langevin et al., 2005). We found that knocking down the expression of exocyst components resulted in a similar stub bristle phenotype,

suggesting that is also involved in chitin deposition. Our data also establish that the intracellular transport of Rab11 in growing bristles is dependent on microtubules.

MATERIALS AND METHODS

Fly culture and strains

All flies were grown on standard media. Oregon R was used as a wild-type control. The *kkv*, *Rab11* and *dyl* mutant and deficiency lines were obtained from the Bloomington *Drosophila* Stock Center (BDSC) at Indiana University. Most of the stocks used for generating flp/FRT somatic clones, *UAS-GFP-Rab11*, *UAS-GFP-Rab11*, *neur-Gal4*, *ap-Gal4* and *ptc-Gal4* were also obtained from the BDSC. The *UAS-Rab11dsRNA.pWIZ* stock was provided by D. Ready (Purdue University); $Rab11^{93Bi}$/TM6, $Rab11^{ex1}$/TM6, $Rab11^{\Delta FRT}$/*TM6*, and *y w hs-flp; FRT5377 hrp-GFP/TM3* by R. Cohen (University of Kansas); *UAS-Klp10A* by T. Uemera (Kyoto University); and UAS-nod-βgal and UAS-kinesin-βgal by Y. N. Jan (University of California, San Francisco). The UAS-*dyl* stocks and the anti-Dyl antibodies were kindly provided by F. Payre (Université Paul Sabatier, Toulouse, France). RNAi stocks for exocyst/*Rab11* and cuticle genes were obtained from the Vienna Stock Center (V lines) and from the BDSC (T lines from the Harvard collection). Key lines for RNAi experiments included V102166 (*dyl*), V22198 (*Rab11*), T27314 (*sec6*), T27483 (*sec10*), T27499 (*sec15*), T28041 (*exo70*), T28712 (*exo84*) and T28874 (sec5). Additional RNAi-inducing transgene lines were used for confirming experiments.

Note that previous studies using the GFP-Rab11 fusion protein refer to it as Rab11-GFP; however, an examination of how the transgene was constructed indicates that Rab11 is fused to the C-terminus of GFP (Emery et al., 2005).

Temperature-controlled gene expression

The GAL4/UAS system was used to direct transgene expression. *neur-Gal4* was used to drive gene expression in bristles. Temporal control of expression utilized a temperature-sensitive Gal80 protein (McGuire et al., 2004). Animals were grown at 21°C, white prepupae collected and pupae shifted to 29°C at the desired stages of development.

Generation of *kkv* and *Rab11* clones

kkv mutant clones were generated by crossing *w hs-flp*; *FRT82 ubi-gfp/TM6* females to FRT82 kkv^{1}/TM6 males. Vials were heat shocked at 37°C for 1 hour to induce *flp* and clone formation. $Rab11^{ex1}$ clones were induced in an analogous experiment. $Rab11^{\Delta FRT}$ clones were induced as described (Bogard et al., 2007).

Immunostaining

Immunostaining was performed by standard protocols on paraformaldehyde fixed material (see He et al., 2005). Primary antibodies used were: rabbit anti-GFP (1:2000, Molecular Probes), mouse anti-acetylated tubulin (1:1000, Sigma), rabbit anti-β-galactosidase (1:10,000, Cappel) and anti-Dyl (1:200, F. Payre). Alexa 488- and Alexa 568-conjugated secondary antibodies (1:250) were from Molecular Probes. For F-actin staining, we used Alexa Fluor phalloidin (488 or 568, Molecular Probes).

Chitin staining using CBD-Rhodamine probe

To visualize chitin in bristles, fixed thoraces were incubated in a 1:100 dilution of Rhodamine-conjugated chitin-binding probe (New England Biolabs) (Gangishetti et al., 2009) in PBS containing 0.3% Triton X-100 for 4 hours at room temperature, rinsed in PBS and mounted. Similar results were obtained using labeled wheat germ agglutinin, although with this reagent it was better not to add detergent to permeabilize the cells.

Inhibition of cytoskeleton using drugs and genetics

Thoraces from pupae at 40-42 hours after puparium formation (APF) (25°C) were cultured in Schneider's medium (1×) in the presence of drugs prior to fixation and staining. We also used the timed expression of *UAS-Klp10A* to disassemble microtubules. Klp10A is a member of the kinesin-13 family that binds to the minus ends of microtubules and induces their depolymerization (Ems-McClung and Walczak, 2010; Heald, 2004) and it has been found to be an effective mediator of microtubule breakdown in several tissues/developmental stages in *Drosophila* and other systems (Ems-McClung and Walczak, 2010; Goodwin and Vale, 2010; Goshima and Vale, 2005; Heald, 2004; Schimizzi et al., 2010; Sharp et al., 2005; Shimada et al., 2006). We found that Klp10A reduced the density of bristle microtubules, as detected by transmission electron microscopy, to ~40% of wild-type levels. This might be an underestimate as microtubule density decreases as bristles elongate (Tilney et al., 2000a) and elongation is severely inhibited by Klp10A expression.

Disruption of chitin synthases using nikkomycin

Nikkomycin (5 mg/ml in water; Sigma) was injected into pupal heads between 28 and 32 hours APF (25°C) using a microinjection apparatus. The drug-treated pupae were compared with water-injected controls.

Live imaging studies

Animals were placed on double-sided sticky tape and part of the pupal case was removed to provide a window that exposed the head and thorax. A coverslip coated with a small volume of halocarbon oil (Sigma) was placed onto the pupa with both sides supported by a piece of rubber. Pupae were imaged every 3-4 hours at 20× and 60× while being incubated at 29°C between imaging sessions. Confocal observations were made on a Nikon Eclipse TE200 microscope equipped with a CARV spinning disc confocal attachment (ATTO). The FRAP and in vivo imaging experiments were performed on a BioRad Radiance 2100 confocal and a Zeiss 510 Meta confocal microscope at the Keck Center for Cellular Imaging at the University of Virginia.

Image analysis

Confocal images were obtained using a CARV spinning disc unit on a Nikon Eclipse TE200 microscope or on a Zeiss Meta confocal microscope. Cuticle images were obtained using a SPOT digital camera (National Diagnostics) on a Zeiss Axioskop 2 microscope. Images were analyzed and scale bars added using ImageJ and processed using Adobe Photoshop.

RESULTS

Rab11 and bristle development

We induced unmarked clones of two different *Rab11* null alleles – $Rab11^{\Delta FRT}$ and $Rab11^{ex1}$ (Bogard et al., 2007; Dollar et al., 2002) – and observed missing bristles. This was expected because *Rab11* is required for the formation of the bristle sense organ lineage (Emery et al., 2005). We also observed occasional abnormal bristles on these flies that were shortened, malformed (Fig. 1B,C) or reduced to stubs (Fig. 1D). We suggest that perdurance of the *Rab11* gene product allowed these bristles to bypass the Rab11 requirement for bristle determination. These phenotypes partly overlapped those seen in *Rab11* hypomorphs ($Rab11^{93Bi}$/$Rab11^{ex1}$). The hypomorphs showed a weaker bristle phenotype that was mostly obvious in macrochaetae, where the bristles were shorter than wild type and had tips that were either blunt or very thin and wavy (Fig. 1E,F). Interestingly, the distal parts of the *Rab11* hypomorph bristles often lacked pigmentation and were almost transparent (Fig. 1E,F, arrows).

We found that expressing *Rab11* dsRNA after the determinative cell divisions was able to produce a much stronger bristle phenotype than the hypomorph. The strength of the mutant phenotype was proportional to the duration of RNAi induction and the particular RNAi-inducing transgene used (Fig. 1G-I). The strongest phenotype resulted in bristles that were reduced to short stubs (Fig. 1I). Such phenotypes were seen with close to complete cellular penetrance for the scutellar macrochaetae. It was possible to obtain very strong phenotypes in all bristles with the appropriate choice of transgenes and temperature shifts (supplementary material Fig. S1).

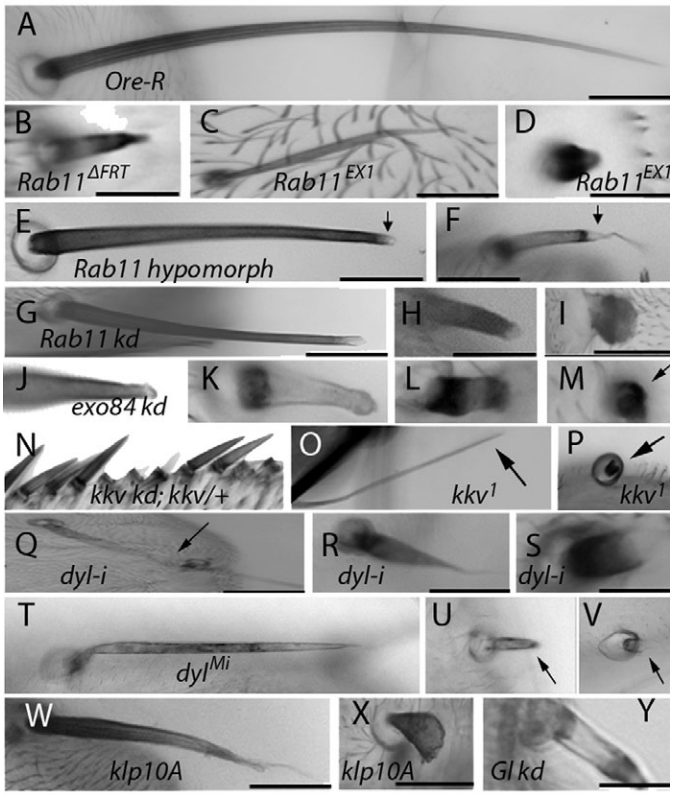

Fig. 1. Bristle phenotypes. (**A**) Scutellar bristle from wild-type (*Ore-R*) adult. (**B**) Thoracic bristle from putative $Rab11^{\Delta FRT}$ null clone. (**C**) Thoracic bristle from putative $Rab11^{ex1}$ null clone. (**D**) Abdominal bristle from putative $Rab11^{ex1}$ null clone. (**E**,**F**) Thoracic bristles from *Rab11* hypomorph ($Rab11^{93Bi}/Rab11^{ex1}$) adults. Arrows point to pigmentation defect at the tip. (**G-I**) *Rab11* knockdown (kd) thoracic bristles. Knockdown starting 19 hours APF (G), 12 hours APF (H), or at white prepupal stage (I). (**J-M**) *exo84* kd bristles (arrow points to bristle in M). (**N**) Wing bristles from flies with disrupted *kkv* function (*kkv RNAi; kkv/+*). (**O**,**P**) Scutellar bristles from putative kkv^{1} clones (arrows point to bristles). (**Q-S**) Thoracic bristles from *dyl* kd (*w; dyl RNAi/+; neur-Gal4/+*). Arrow points to affected bristle in Q. (**T-V**) Bristles from a $dyl^{ML02088}$ escaper fly (arrows point to bristles). (**W**,**X**) Thoracic bristles from flies expressing Klp10A. Expression starting 15 hours APF (W) or at white prepupal stage (X). (**Y**) Thoracic bristle from fly with $p150^{glued}$ kd from white prepupal stage to adult. All images were taken at 10× magnification. Scale bars: 100 μm.

As a test of the functionality of a GFP-Rab11 fusion protein, we constructed flies that were *UAS-GFP-Rab11/+; $Rab11^{93Bi}$ neur-Gal4/$Rab11^{ex1}$*. The hypomorphic bristle phenotype in such flies was completely rescued both in length (supplementary material Fig. S2) and morphology, confirming that the GFP-Rab11 protein was functional. To test whether GFP-Rab11 was intact we analyzed extracts of wing discs from *UAS-gfp-Rab11/ptc-Gal4* larvae by western blotting. A single band of the expected size was detected (supplementary material Fig. S3). We concluded that GFP-Rab11 was intact, functional and an accurate reporter for Rab11.

The accumulation of an organizing/marking protein at a particular region of the plasma membrane is often a crucial step in the growth of polarized cell structures (Pruyne et al., 2004; Tang, 2001). Since *Rab11* mutants showed a strong bristle phenotype with altered tip morphology, we examined the localization of GFP-Rab11 in developing bristles by immunostaining and by direct in vivo imaging. It was most abundant in the cell body and punctate localization was observed in the shaft (Fig. 2A-F, Fig. 3A,B). Interestingly, the tagged Rab11 was enriched at the distal tip of growing bristles throughout the period of bristle elongation. This was also reported by Hayashi and colleagues (Otani et al., 2011). The GFP-Rab11 at the tip was distal to the large bundles of F-actin (Fig. 2D-F), suggesting that it could have an organizing function, similar to that which Rab11 is thought to have in the oocyte for microtubules (Dollar et al., 2002). Recent FRAP experiments by Otani et al. showed Rab11 movement and localization to be very dynamic in growing bristles (Otani et al., 2011), a result we independently obtained.

If Rab11 accumulation at the tip is required for polarized growth, we predicted that it should initiate at very early stages in bristle development. When bristle outgrowth begins, the shaft cell is located below the socket cell, making it difficult to reliably analyze protein localization. We therefore examined the lateral branches of the arista for GFP-Rab11 localization. The cytoskeleton of elongating laterals is similar to that of bristles, but they differ in that the initial site of outgrowth is not covered (He and Adler, 2001). At the time of initiation of outgrowth (28 hours APF, 25°C) a crescent of GFP-Rab11, often with a small blob-like protrusion, formed on the plasma membrane at the site of outgrowth (Fig. 2G-I). F-actin also accumulated at this site (Fig. 2H,I) but GFP-Rab11 appeared to be more focused at the tip than the actin (Fig. 2I). Four hours later, when extension had started, the tip localization of GFP-Rab11 was retained and the shaft also showed localization (Fig. 2J-L). As observed in growing bristles, the tip localization of Rab11 was distal to the actin bundles.

Studies in mammalian cells and *Drosophila* have asserted the importance of both actin filaments and microtubules in the Rab11-mediated transport of proteins (Casanova et al., 1999; Dollar et al., 2002; Hales et al., 2001; Hales et al., 2002; Lapierre et al., 2001; Leung et al., 2000; Satoh et al., 2005). It has been suggested that the microtubules play a key role in transporting proteins required for bristle growth (Fei et al., 2002; Tilney et al., 2000a), although experimental evidence for this is lacking.

We first tested the role of the cytoskeleton in GFP-Rab11 localization by culturing pupal thoraces in the presence of drugs that are known to disrupt actin or microtubules. Disruption of the microtubule cytoskeleton had a greater effect on GFP-Rab11 distribution than disruption of the actin cytoskeleton (supplementary material Fig. S4), but these experiments were not definitive. Therefore, as an alternative method for inhibiting microtubule function, we used the GAL4/UAS/$Gal80^{ts}$ system to express Kinesin-like protein (KLP) 10A in growing bristles (see Materials and methods). Klp10A induces microtubule depolymerization (Goshima and Vale, 2005; Heald, 2004; Sharp et al., 2005; Shimada et al., 2006).

Using time-lapse imaging, we tracked bristle development in pupae expressing GFP-Rab11 and Klp10A as compared with those expressing only GFP-Rab11. As observed previously, GFP-Rab11 localized to the bristle tip as early as 28 hours APF in the control bristles (Fig. 3A). The localization at the tip and in the shaft increased over the next 8 hours (Fig. 3B-D). Interestingly, the tip localization decreased after the elongation phase, although GFP-Rab11 remained abundant throughout the shaft (Fig. 3D). By comparison, GFP-Rab11 localization in the Klp10A-expressing bristles was dramatically reduced, both in the shaft and the bristle tip (Fig. 3E-H), but not in the cell body. We concluded that Rab11 moves from the cell body to the growing shaft in a microtubule-dependent manner.

DEVELOPMENT

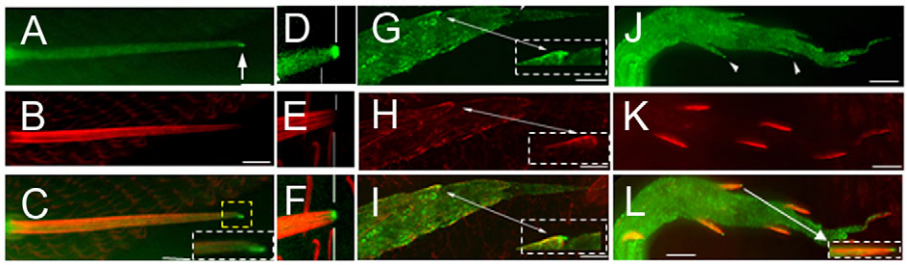

Fig. 2. GFP-Rab11 localization in bristles and laterals. GFP-Rab11 (green) localization was visualized by immunostaining with anti-GFP antibody. F-actin (red) was visualized by phalloidin staining. (**A**) GFP-Rab11 is found in the bristle cell body and shaft. It is enriched at the tip (arrow). (**B**) F-actin for the bristle in A. (**C**) Merge of A and B. The tip is magnified in the inset. (**D-F**) Localization of GFP-Rab11 with respect to actin filaments. (D) GFP-Rab11 is enriched at the tip (arrow). (E) F-actin for bristle shown in D. (F) Merge of D and E. Note that Rab11 at the tip is distal to the F-actin. (**G-I**) Early stages of lateral outgrowth (28 hours APF, 25°C). (G) GFP-Rab11. (H) F-actin. (I) Merged image. Note the very early tip enrichment of GFP-Rab11. Insets are magnifications of the indicated regions (arrowed). (**J-L**) Middle stage of lateral outgrowth (32 hours APF, 25°C). (J) GFP-Rab11. Arrowheads point to Rab11 at tip. (K) F-actin. (L) Merged image. All images were taken at 60× magnification. Scale bars: 10 μm.

In the *Drosophila* oocyte the posterior accumulation of Rab11 is required for polarizing microtubules so that plus ends are preferentially located in the posterior (Dollar et al., 2002). We speculated that Rab11 could be performing a similar role in bristles to mediate the polarized transport of protein(s) and membrane to the tip. To test this hypothesis, we analyzed microtubule polarity in bristles by expressing reporter proteins. Both Kin-βgal and GFP-Nod (Cui et al., 2005; McDonald et al., 1990) are plus-end reporters and they localized to the bristle cell body (supplementary material Fig. S5A,B). By contrast, the Nod-βgal fusion protein is a minus-end reporter (Clark et al., 1997; Cui et al., 2005) and it localized to the distal tip (supplementary material Fig. S5C). These observations indicated that microtubules are polarized in bristles with their minus ends

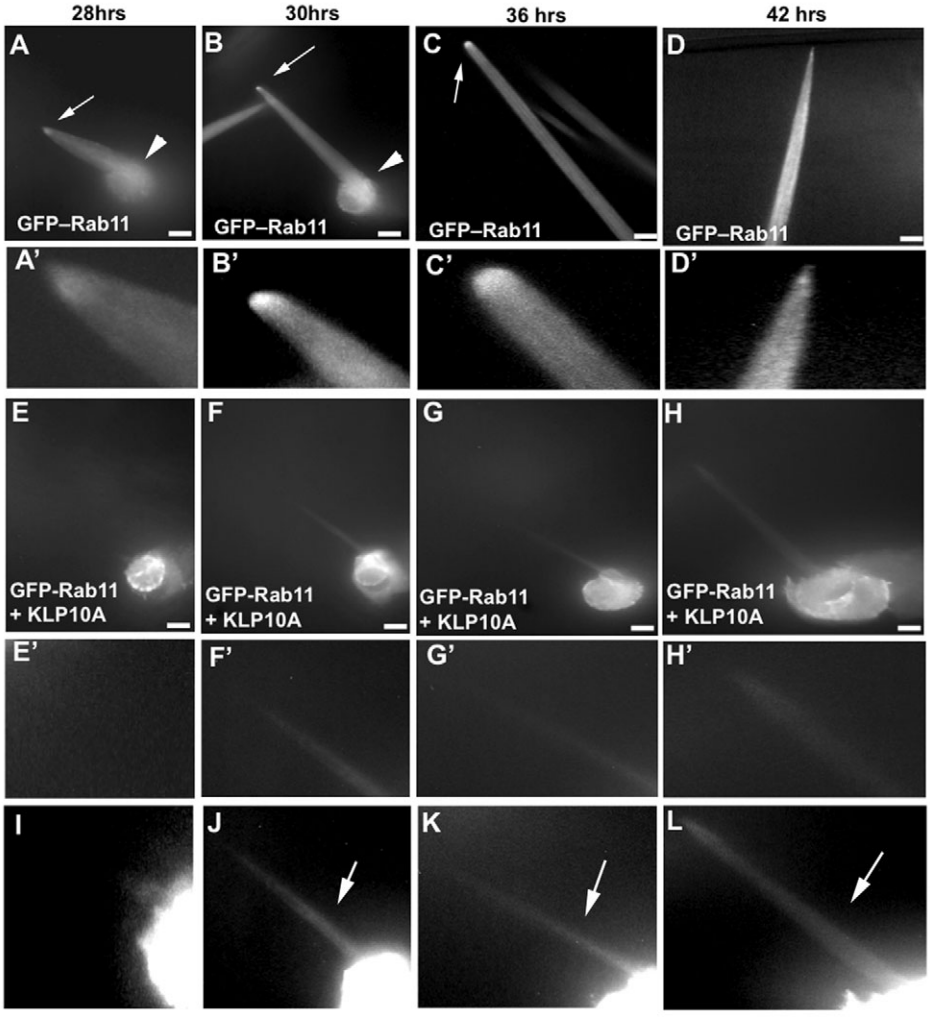

Fig. 3. Rab11 intracellular transport is microtubule dependent. Time-lapse imaging of bristles expressing GFP-Rab11 and Klp10A under *neur-Gal4* as compared with control bristles expressing only GFP-Rab11. (**A-C**) GFP-Rab11 localization in a wild-type bristle cell during its growth phase. Note the tip enrichment of Rab11 (arrows). Note the cell body GFP-Rab11 (arrowheads). (**D**) GFP-Rab11 localization in a wild-type bristle cell near the end of its growth phase. Note the decreased tip enrichment. (**A'-D'**) Magnified images of the bristle tip from A-D, respectively. (**E-G**) GFP-Rab11 localization in a bristle expressing Klp10A during its growth phase (starting ~24 hours APF). (**H**) GFP-Rab11 localization in a bristle expressing Klp10A after its growth phase. (**E'-H'**) Magnified images of the bristle tip from E-H, respectively. (**I-L**) Highly enhanced versions of images E-H that allow the bristle shaft structure (arrows) to be seen. Images are of a single bristle tracked in each set. Images were taken at 60× magnification and represent a projection of optical sections. In control images C and D, the entire length of the bristle cell is not shown. Scale bars: 10 μm.

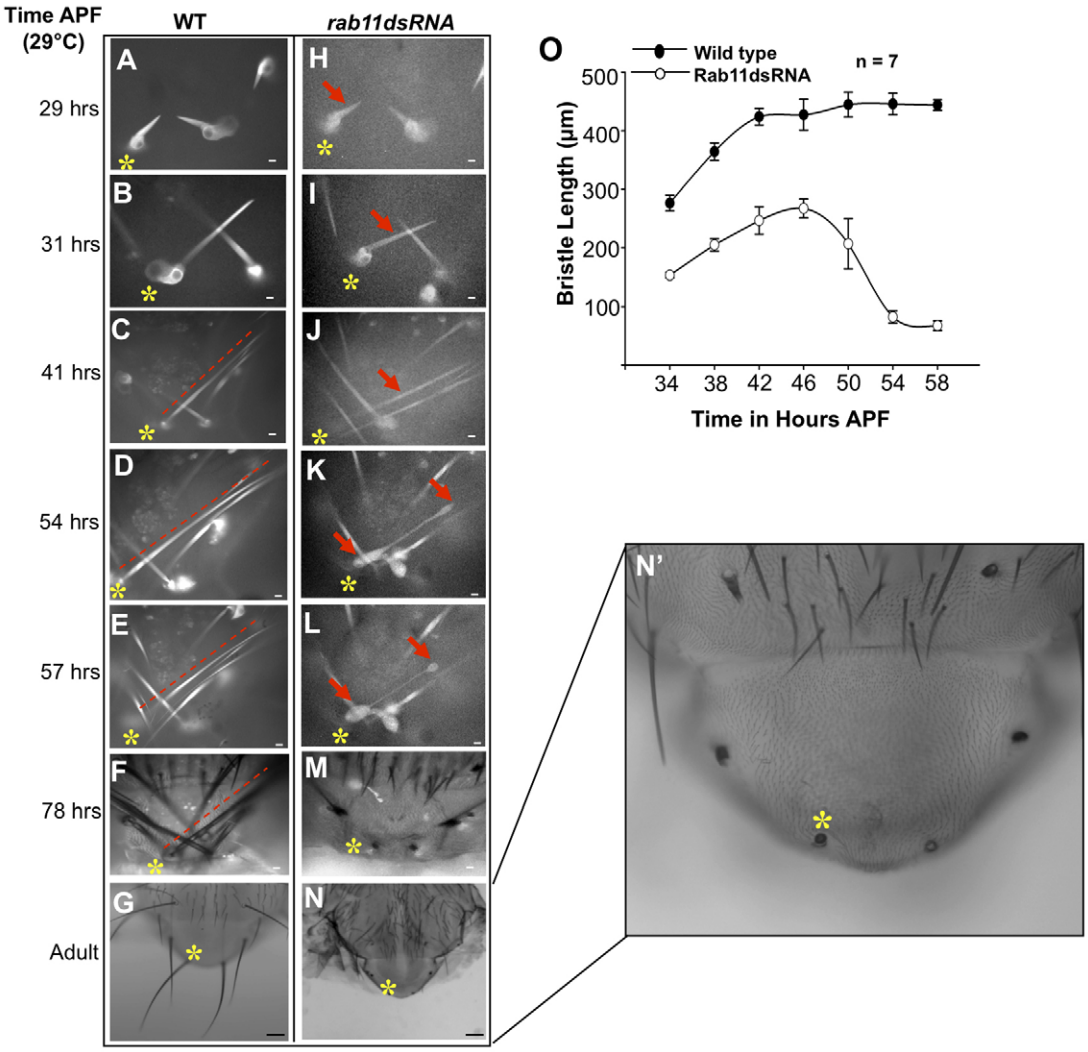

Fig. 4. Rab11 is essential for bristle stability. (**A-N'**) Time-lapse imaging of wild-type and *Rab11* dsRNA-expressing bristles. All images except G and N (2.5×) were taken at 20× (oil immersion) magnification. Images are shown of the same pupa tracked from 29 hours APF to adult. Asterisks and arrows mark the bristle being tracked; dashed line is parallel to the bristle. Images represent single optical sections. (A-G) Wild-type pupal thoracic bristles (control). (H-N') *Rab11* kd bristles (kd starting at white prepupal stage). Arrows in K and L indicate regions of bristle that showed deformities. (N') Enlargement of N. Scale bars: 10 µm in A-F,H-M; 100 µm in G,N. (**O**) The growth of wild-type and *Rab11* dsRNA-expressing bristles over time as determined by measuring the length of bristles (µm) at different times of development (hours APF at 29°C). Mean ± s.d.

toward the distal tip and plus ends toward the cell body. Similar results on the polarity of microtubules were reported by Bitan et al. (Bitan et al., 2010). Surprisingly, the relationship between Rab11 and microtubule ends is reversed in bristles as compared with oocytes (Clark et al., 1997; Dollar et al., 2002). We found the Nod-βgal and Kin-βgal reporters to be very toxic. Even a short period of expression of Nod-βgal led to bloated bristle tips (supplementary material Fig. S5A). In adult bristles this treatment led to bristles with a variety of tip abnormalities, including reduced pigmentation, thin wispy shafts and swollen tips (supplementary material Fig. S5E). By contrast, expression of Kin-βgal led to bristles that were frail and thinned (supplementary material Fig. S5F,G). We suggest that these polarity reporters act as dominant-negative motors and block transport along microtubules, which leads to their accumulating in a polarized fashion. The toxic nature of the microtubule polarity reporters made it difficult for us to use them to reliably analyze a possible role for Rab11 in bristle microtubule organization.

Rab11 is important for chitin deposition and bristle stability

We hypothesized that the phenotypes displayed by *Rab11* mutants were due to a defect in bristle elongation. To determine whether this was the case, we performed live imaging studies in pupae expressing *Rab11* dsRNA as well as wild-type controls (Fig. 4). *neur-Gal4>*Tubulin-GFP was used for imaging developing bristles. *Rab11* dsRNA-expressing bristles were shorter and grew more slowly than control bristles in the growth phase (29-42 hours APF) (Fig. 4O). Nevertheless, they did elongate and by 46 hours APF reached 260 µm in length, which was more than half the length of controls (Fig. 4I,J). In addition to growing more slowly, bristles from *Rab11* dsRNA-expressing pupae also started displaying deformities/bulges at later stages (54-57 hours APF; Fig. 4K,L). These deformities worsened over time and led to the collapse of the bristle (Fig. 4M,O).

We concluded that the dramatic stub phenotype was not due to a failure of bristle outgrowth but rather represented what was left after bristle collapse. Examining numerous bristles we observed

that the details of the deformities and collapse were highly variable. That is, the regions where the bulges first arose and the path of changes that preceded the collapse were not consistent. However, we first observed the deformities at ~48 hours APF regardless of their initial location along the shaft (Fig. 4K,L). We also examined bristles from pupae expressing *Rab11* dsRNA for a shorter period of time (supplementary material Fig. S6). The adult bristles from such flies displayed weaker bristle phenotypes (supplementary material Fig. S6A-G). In such cases, we found that bristles elongated to a greater extent and then snapped, broke or collapsed, leaving behind a shorter bristle with an abnormal tip (supplementary material Fig. S6E-G).

The exocyst is known to be a Rab11 effector (Emery et al., 2005; He and Gou, 2009; Langevin et al., 2005) and Sec5 colocalizes with Rab11 in growing bristles (Otani et al., 2011). We tested seven of the eight exocyst components (*sec5*, *sec6*, *sec8*, *sec10*, *sec15*, *exo70* and *exo84*) for a function in bristle morphogenesis using the same timed RNAi knockdown (kd) we used for *Rab11*. For all seven we observed bristle abnormalities that spanned the range seen with *Rab11*, including the stub phenotype (Fig. 1J-M). The frequency of exocyst kd-induced stub phenotypes was, however, routinely lower than that seen for *Rab11*, and *UAS-Dicer-2* was needed as an RNAi enhancer (Dietzl et al., 2007). In our best experiments, ~75% of the scutellar bristles displayed the stub phenotype. We did not carry out time-lapse in vivo imaging experiments on exocyst kd bristles, but observations of fixed 42-hour pupae indicated that the adult stub bristle phenotype was due to collapse. Consistent with the exocyst functioning in Rab11-independent processes, lethality was routinely more extensive with exocyst kd. Our results suggest that the exocyst is a Rab11 effector during bristle development.

Our experiments showed that bristle collapse occurred late in development (Fig. 4O), around the time when actin filament breakdown is initiated (Guild et al., 2002). We therefore examined actin filaments in bristles before and at the time of collapse to determine whether actin breakdown was affected in the *Rab11* kd bristles. We did not detect any change in the timing of actin filament breakdown due to *Rab11* kd (supplementary material Fig. S7). We concluded that the collapse was not mediated by an effect on F-actin.

The correlation between the timing of actin filament breakdown and the collapse of *Rab11* kd bristles suggested that collapse was associated with a failure in a component that provided structural integrity after actin bundle breakdown. The cuticular exoskeleton was a likely candidate; therefore, we examined the deposition and accumulation of chitin in wild-type and *Rab11* dsRNA-expressing bristles. Chitin is a major component of cuticle but the timing and arrangement of chitin deposition in *Drosophila* bristles has not been reported. We first detected chitin at ~42 hours APF. It was first seen proximally and shortly thereafter all along the shaft bristle (Fig. 5A,A′). As development proceeded, chitin was found in increasingly distinct bands that ran parallel to the long axis of the bristle (Fig. 5E,E′,I,I′). When we analyzed *z* projections we observed that chitin was external to the bundles of F-actin and covered the entire bristle circumference (Fig. 5M,N). The banded pattern seen in optical sections and maximal projections is likely to be a consequence of the fluted shape of the bristles and variation in chitin thickness. As expected for an extracellular component (Moussian et al., 2005), we were able to stain bristle chitin in the absence of permeabilization with detergent (supplementary material Fig. S8A).

Rab11 kd bristles displayed dramatically altered chitin banding. The abnormalities ranged from delayed deposition (Fig. 5C), bands that did not run parallel to the long axis of the bristle, to regions with a complete loss of chitin (Fig. 5G′,K′). These abnormalities were usually more severe in older bristles, suggesting that there was not only a defect in the deposition of chitin but also in its stability. In *z* projections, large chitin-free regions were often seen (Fig. 5O,P). In addition to the disorganization there was also a quantitative decline in the amount of chitin staining and this also became more severe at later stages (supplementary material Fig. S9).

In *Drosophila* and other insects there are two chitin synthase genes. CS-1 is encoded by *krotzkopf verkehrt* (*kkv*) and mediates cuticle formation in the epidermis and trachea in embryos, larvae and adults (Coutinho et al., 2003; Merzendorfer and Zimoch, 2003; Ostrowski et al., 2002; Ren et al., 2005; Roncero, 2002; Zimoch and Merzendorfer, 2002). The second chitin synthase (CS-2) is thought to only function in the synthesis of the peritrophic membrane. As null alleles of *kkv* are embryonic lethal, to examine its function in bristle development we generated somatic clones of kkv^{1} [a null allele (Ostrowski et al., 2002)]. Flies carrying large or numerous clones often died as pharate adults due to an apparent defect in the integrity of the cuticle barrier. Flies with few or smaller clones often eclosed. In the abdomen of such flies, clones were often associated with ‘blobs of exudates’ (supplementary material Fig. S10C). The clone cuticle had reduced pigment and trichomes were faint as expected (supplementary material Fig. S10A) (Ren et al., 2005). Bristle abnormalities ranged from rare very short bristles to frequent moderately short bristles with abnormal pigmentation (Fig. 1N-P). Although there was overlap between the kkv^{1} and *Rab11* kd phenotypes, the kkv^{1} mutant bristles generally showed a substantially weaker phenotype, suggesting that Rab11 function is likely to be required for the deposition of multiple cuticular components. A similar range of phenotypes was seen in experiments in which we injected the chitin synthase inhibitor nikkomycin into pupae (supplementary material Fig. S10D-L) (R. Nagaraj, PhD Thesis, University of Virginia, 2010).

Dusky-like functions as a Rab11 effector

The formation and maintenance of chitin is a complex process that requires the function of chitin synthases, chitin-binding proteins and chitinases (Merzendorfer and Zimoch, 2003). We identified *dusky-like* (*dyl*) as a compelling candidate for functioning in bristle cuticle formation. Dyl is a ZP domain-containing transmembrane protein that is expressed only in cuticle-secreting epithelia such as the epidermis, trachea and foregut (Fernandes et al., 2010; Hillman and Lesnick, 1970; Roch et al., 2003). Knocking down *dyl* function in bristles resulted in a range of bristle phenotypes (Fig. 1Q-S) that were similar to those seen with *Rab11*. Most notable were the stub phenotype and pigmentation abnormalities (Fig. 1Q-S). We found that heterozygosity for a deficiency that removed *dyl* strongly enhanced the weak scutellar bristle phenotype that resulted from *dyl* kd using either the *ptc-Gal4* or *sca-Gal4* drivers (supplementary material Fig. S11F). Recently, a stock carrying a Minos insertion into *dyl* became available. This mutation is recessive lethal, with most homozygotes dying as prepupae or young pupae. Rare escapers showed bristle phenotypes that were similar to those seen in the kd experiments (Fig. 1T-V). Hence, we concluded that the *dyl* RNAi phenotype was specific. As with *Rab11*, we observed alterations in chitin band deposition in *dyl* kd bristles (Fig. 5Q).

Similarities between bristle phenotypes suggested that Dyl might regulate Rab11 function (or vice versa) to mediate chitin deposition in bristles. To determine whether *dyl* is required for Rab11 localization we examined GFP-Rab11 by live imaging in *dyl* kd bristles. These experiments showed that *dyl* kd bristles grew and

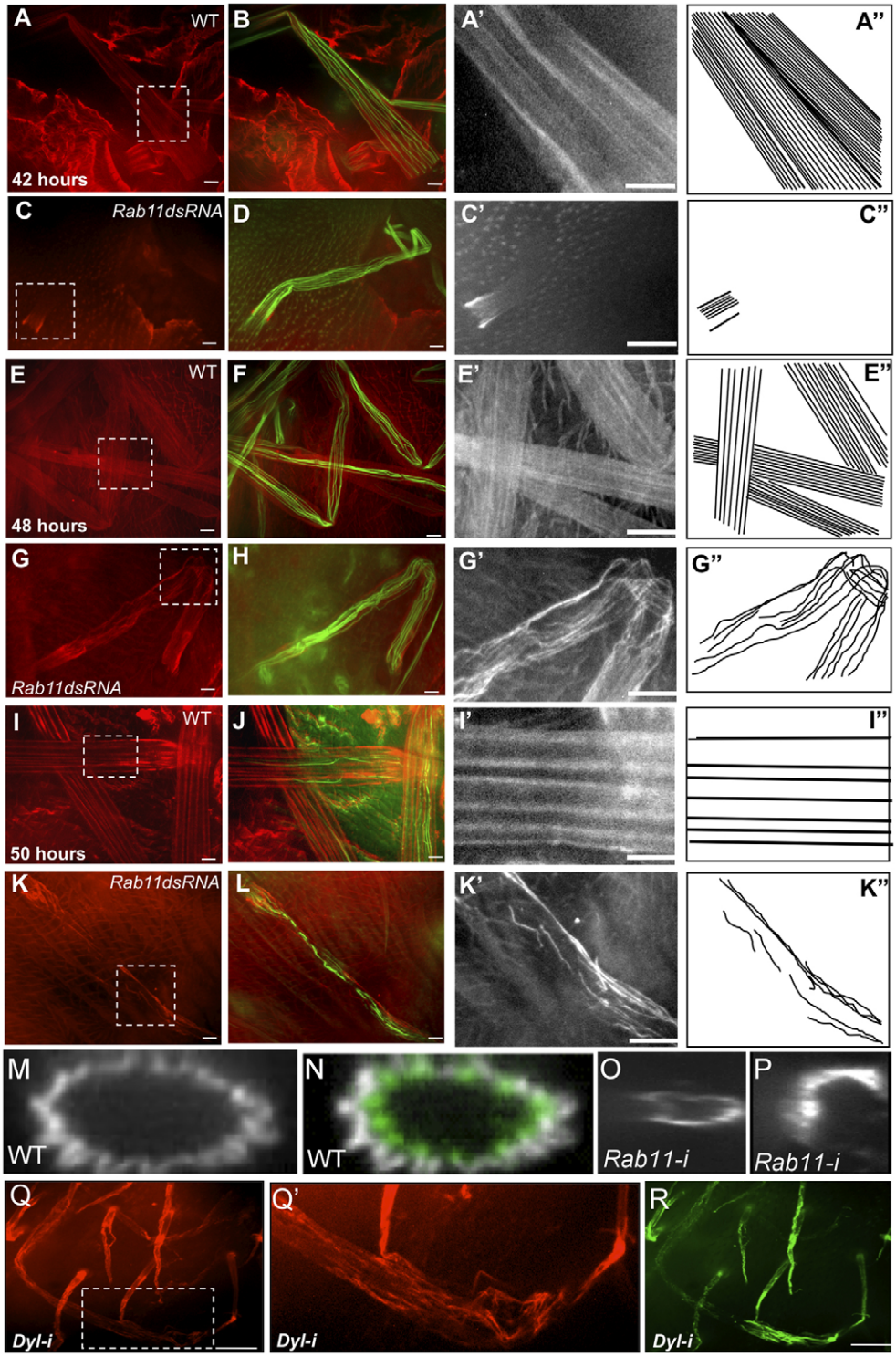

Fig. 5. Chitin organization in bristles. (**A-L**) Chitin (red) and actin (green) in wild-type bristles and *Rab11* kd bristles at 42, 48 and 50 hours APF. Magnifications of chitin staining (boxed regions) are shown in grayscale in A′,C′,E′,G′,I′,K′, and interpretive drawings of these in A″,C″,E″,G″ I″,K″. (**M**,**N**) Projection of *z*-stacks from images of wild-type bristles stained for chitin (grayscale) and F-actin (green) localization. (**O**,**P**) Projection of *z*-stacks of *Rab11* kd bristles showing uneven chitin deposition along the bristle circumference. (**Q-R**) Chitin (red) and F-actin (green) in *dyl* kd bristles. (Q′) Enlargement with enhanced contrast of boxed region from Q. Scale bars: 10 μm.

then collapsed like the *Rab11* mutant bristles (Fig. 6A-C). Disrupting *dyl* function did not affect the tip localization of GFP-Rab11 (Fig. 6A,B, arrow). Normal Rab11 distribution was still evident when morphological abnormalities first appeared (Fig. 6). As the deformities worsened, GFP-Rab11 accumulated in the 'bulges' of collapsing bristles (Fig. 6C). We suggest that the late alteration in GFP-Rab11 localization was due to the changes in bristle morphology and was not a direct consequence of disrupting *dyl* function.

To determine whether *Rab11* functions upstream of *dyl* we examined Dyl localization by immunostaining (Fernandes et al., 2010) in both wild-type and *Rab11* kd bristles. In wild-type bristles, Dyl was localized in parallel stripes along the length of the shaft (Fig. 6D′) that were off set from the bundles of F-actin. In *z* sections, Dyl was at the periphery and external to the actin bundles. (Fig. 6J-L). Since Dyl is a transmembrane protein, we concluded that Dyl was localized to the plasma membrane of bristles. In *Rab11* kd bristles, Dyl was not found in stripes and in *z* sections it

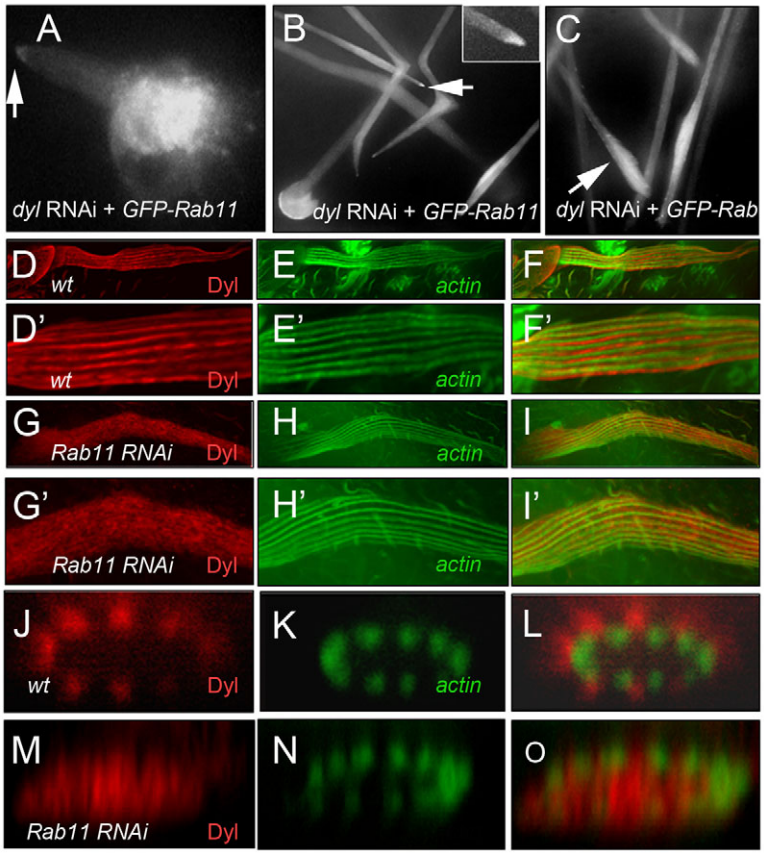

Fig. 6. Dyl and Rab11 localization in bristles. (**A-C**) GFP-Rab11 localization in bristle expressing *dyl* RNAi at (A) 29 hours, (B) 40 hours and (C) 56 hours APF at 29°C. Arrows in A and B indicate tip accumulation of GFP-Rab11 (magnified in inset in B). Arrow in C shows the accumulation of GFP-Rab11 in a blebbing region of a bristle. (**D-O**) Dyl localization in wild-type and *Rab11* mutant bristles visualized by staining with anti-Dyl antibody. Localization of (D) Dyl and (E) F-actin in wild-type bristles. (F) Merge of D and E. (D′-F′) Magnifications of D-F, respectively. (G,H) Localization of Dyl (G) and F-actin (H) in *Rab11* kd bristles. (I) Merge of G and H. (G′-I′) Magnifications of G-I, respectively. (J-O) *z* projections showing Dyl (J,M) and F-actin (K,N) localization around the circumference of wild-type (J,K; merge in L) and *Rab11* kd (M,N; merge in O) bristles.

was spread throughout the cytoplasm and not restricted to the cell periphery. Thus, Rab11 function is essential for localizing Dyl to the bristle plasma membrane.

Microtubule function is essential for bristle elongation

Expressing Klp10A resulted in mutant phenotypes that were similar to those of *Rab11* mutants. Bristles similar to those seen in *Rab11* hypomorphs (Fig. 1W) were seen when Klp10A was induced at 15 hours APF. When Klp10A was induced from the white prepupal stage onwards, we observed bristles that resembled the strong *Rab11* kd stub phenotype (Fig. 1X). Previous studies with microtubule antagonists such as vinblastine produced similar phenotypes (Fei et al., 2002).

We carried out in vivo imaging to determine the basis for the Klp10A stub phenotype. In contrast to the results seen for *Rab11*, the expression of Klp10A resulted in a failure of bristle extension (Fig. 7). Such bristles were shorter than those of the wild type at all stages and no collapse was detected. These observations showed that, although the adult bristle phenotypes of *Rab11* mutants resemble those from flies in which Klp10A was expressed, the mechanisms that gave rise to the phenotypes were very different.

The Klp10A phenotype could be a direct consequence of the reduced number or size of microtubules or, alternatively, it could be due to an indirect effect on one or more microtubule-dependent processes such as long-distance intracellular transport. To distinguish between these possibilities, we examined bristles in which $p150^{glued}$ (Glued – FlyBase) function was compromised. $p150^{glued}$ is the largest subunit of the dynactin complex and serves as an adaptor to facilitate the binding of cytoplasmic dynein to its cargo and to meditate the interaction between dynein and microtubules (Gill et al., 1991).

Glued function was knocked down in bristle-forming cells in pupae. The strongest phenotypes we observed resembled the *Rab11* hypomorphic phenotype (Fig. 1E,F). The *Glued* kd bristles were shorter than normal, had almost transparent tips and showed excess pigment just proximal to the tip (Fig. 1Y). We concluded that dynactin-mediated intracellular transport on microtubules is important for bristle extension and pigmentation and that a disruption of intracellular transport is likely to be the basis for the Klp10A phenotype.

DISCUSSION

Dyl functions as a Rab11 effector

We found that knocking down *Rab11* function in bristles led to short stubs. Very similar bristle stub phenotypes were observed when any of seven different exocyst components or Dyl function was knocked down. In vivo imaging showed that the bristle stub phenotype was not due to a primary failure in bristle elongation. Rather, the phenotype resulted from the collapse of bristles at the time when cuticle takes over the structural support role from F-actin bundles. We documented highly abnormal chitin deposition in both the *Rab11* and *dyl* kd, consistent with a failure of cuticle formation being the cause of the collapse.

An analogous role for Rab11 has been observed previously in *S. cerevisiae*, where the yeast homologs Ypt31 and Ypt32 spatially and temporally regulate the delivery of Chs3p (one of the yeast chitin synthases) to the plasma membrane to mediate chitin deposition (Ortiz and Novick, 2006). This suggests that Rab11 regulates chitin formation in bristles by mediating the transport and localization of chitin-synthesizing enzymes to sites of cuticle deposition in bristles. If this were true and it was the only function of Rab11 in bristle morphogenesis then disrupting the function of

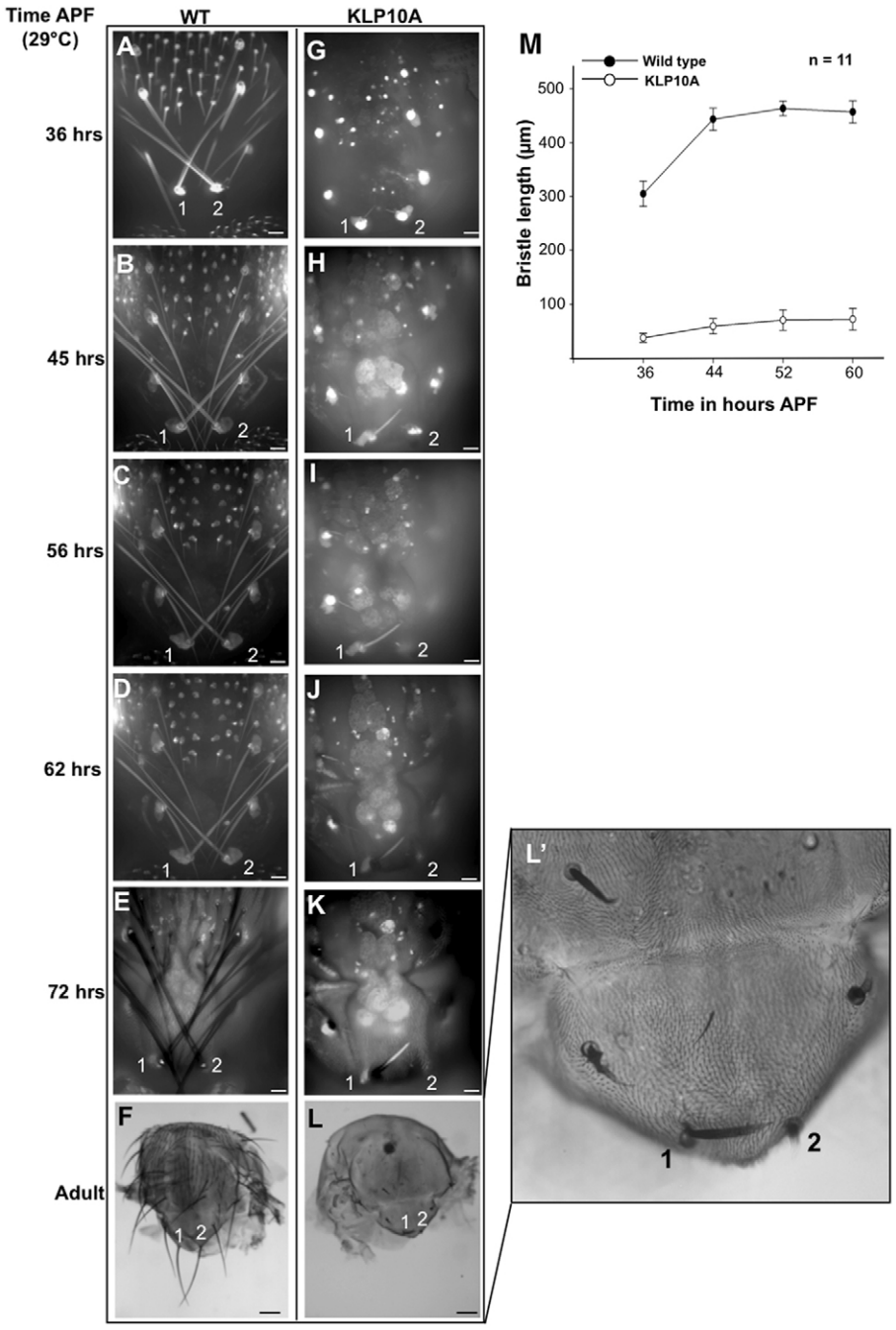

Fig. 7. Microtubules are essential for bristle growth. (**A-L'**) Time-lapse imaging of wild-type and Klp10A-expressing bristles. Images of the pupal bristles and adult bristles were taken at 20× and 2.5× magnification, respectively. (A-F) Wild-type bristles 1 and 2 tracked over 40 hours of development from pupa to adult stage. (G-L) Klp10A-expressing bristles 1 and 2 tracked over 40 hours of development. (L') Magnification of L. Scale bars: 10 μm in A-E,G-K; 100 μm in F,L. (**M**) Growth of wild-type and Klp10A-expressing bristles over time. Mean ± s.d. Images are projections of single optical sections.

chitin synthases in bristles should cause similar phenotypes to those of *Rab11* mutants. However, bristles homozygous for a null allele of *kkv*, which encodes the relevant chitin synthase, had, on average, weaker bristle phenotypes than *Rab11* mutants, suggesting that there are cuticle targets other than chitin synthase that require Rab11 for localization, secretion or organization in bristles. Candidate targets include cuticle proteins and proteins required for cross-linking and pigmenting cuticular structures.

We identified Dyl as such a target. We established that Rab11 is required for the plasma membrane localization of Dyl and that chitin is similarly disorganized in *dyl* and *Rab11* mutants. It is therefore likely that Dyl functions as a Rab11 effector in mediating patterned chitin deposition in bristles. Dyl, like other ZP (zona pellucida) domain-containing proteins is membrane anchored and shares homology with several vertebrate and invertebrate apical matrix components. Genes encoding sixteen ZP domain proteins have been identified in *Drosophila melanogaster*, eight of which have been shown to have highly specific roles in the shaping of embryonic epidermal denticles, where they mediate interactions between the apical extracellular matrix and the epidermal cell membrane (Fernandes et al., 2010). Dyl could therefore function to mediate interactions between chitin and/or other cuticle components and the bristle cell membrane to ensure the proper addition and organization of the cuticle. Interestingly, Dyl was the only ZP domain protein among the four that we examined to affect bristle development: *dusky*, *miniature* and *dumpy* did not show bristle phenotypes. This suggests that ZP domain proteins have cell type-specific functions. This strengthens the possibility that Dyl

functions as a cuticle-specific effector of Rab11 function in bristle cells. *Rab11* presumably has *dyl*-independent functions earlier in bristle development, as *Rab11* is required for the asymmetric cell divisions that give rise to the sense organ (Emery et al., 2005) but we found no evidence for a similar requirement for *dyl*.

Does the exocyst function as a Rab11 effector in bristle cuticle formation?

Based on what is known from other systems, it is expected that any gene(s) encoding a product that is required for the trafficking of Dyl to the plasma membrane or for the secretion of chitin (or chitin synthase) and cuticle proteins would share the *Rab11/dyl* bristle stub phenotype. We observed such phenotypes upon kd of each of the seven exocyst-encoding genes examined, providing strong support for the proposal that the exocyst functions as a Rab11 effector for bristle cuticle deposition. It is interesting that a genome-wide screen failed to identify a role for exocyst component genes such as *sec3*, *sec5*, *sec6*, *sec8* and *sec15* in bristle morphogenesis (Mummery-Widmer et al., 2009). This is due to organismal lethality in the kd, and uncovering the exocyst role in bristle morphogenesis required a temporally limited kd that would be very laborious in a genome-wide screen.

ZP domain proteins and the generation of cuticle diversity

In considering the role of Rab11 and Dyl in bristle morphogenesis it is worth noting that the defect was not simply a quantitative problem with chitin deposition but rather there appeared to be a profound disruption in chitin organization in the mutants. This suggests that Rab11 and Dyl not only function in the secretion of chitin but also serve to pattern the extracellular space for chitin deposition and cuticle formation. Different insect cuticles have different physical properties consistent with their different physiological functions. ZP domain proteins seem likely to play a role in generating this specificity. This appears to be the case in the embryo, where different ZP domain proteins localize to different parts of developing embryonic denticles and mutations in the relevant genes produce distinct phenotypes (Fernandes et al., 2010). In addition, based on their mutant phenotypes it appears that the *dusky* and *miniature* genes, which also encode ZP domain proteins, have an important function in the formation of wing blade cuticle but not in bristle or hair cuticle (Roch et al., 2003). Further studies on this family of genes could provide unique insights into the evolution of cuticle structure and function, which has facilitated the extraordinary diversity of insect morphology.

The distal tip localization of Rab11

We found that GFP-Rab11 preferentially accumulated at the tip of growing bristles. This was also seen by Hayashi and co-workers (Otani et al., 2011), who found that the IKKε kinase was required for the tip localization of Rab11. IKKε is also localized to bristle tips, as is the interacting SpnF protein (Bitan et al., 2010; Otani et al., 2011). Interfering with the function of these genes leads to bristle morphological abnormalities that are nonetheless not as severe as the stub phenotypes we describe here (Abdu et al., 2006; Bitan et al., 2010; Otani et al., 2011; Shapiro and Anderson, 2006). Thus, Rab11 mislocalization appears to create a less serious problem for bristle morphogenesis than a lack of Rab11. These observations suggest that Rab11 function is required all along the growing bristle for Dyl insertion into the membrane and chitin deposition.

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Competing interests statement
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