A transport and retention mechanism for the sustained distal localization of Spn-F–IKKε during Drosophila bristle elongation

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ABSTRACT

Stable localization of the signaling complex is essential for the robust morphogenesis of polarized cells. Cell elongation involves molecular signaling centers that coordinately regulate intracellular transport and cytoskeletal structures. In Drosophila bristle elongation, the protein kinase IKKε is activated at the distal tip of the growing bristle and regulates the shuttling movement of recycling endosomes and cytoskeletal organization. However, how the distal tip localization of IKKε is established and maintained during bristle elongation is unknown. Here, we demonstrate that IKKε distal tip localization is regulated by Spindle-F (Spn-F), which is stably retained at the distal tip and functions as an adaptor linking IKKε to cytoplasmic dynein. We found that Javelin-like (Jvl) is a key regulator of Spn-F retention. In jvl mutant bristles, IKKε and Spn-F initially localize to the distal tip but fail to be retained there. In S2 cells, particles that stain positively for Jvl or Spn-F move in a microtubule-dependent manner, whereas Jvl and Spn-F double-positive particles are immobile, indicating that Jvl and Spn-F are transported separately and, upon forming a complex, immobilize each other. These results suggest that polarized transport and selective retention regulate the distal tip localization of the Spn-F–IKKε complex during bristle elongation.

KEY WORDS: Cell elongation, Cell polarity, IKKε, Ik2, Dynein, Drosophila

INTRODUCTION

Highly polarized cells, such as neurons and epithelial cells, rely heavily on intracellular transport mechanisms for their functional differentiation (Apodaca et al., 2012; Maeder et al., 2014). Disrupted intracellular transport systems lead to a variety of diseases, including neurodegeneration and microvillus inclusion diseases (Encalada and Goldstein, 2014; Millencamps and Julien, 2013; Golachowska et al., 2010). Accurate intracellular transport is ensured by the polarized cytoskeleton and by the adaptor protein–mediated recognition of specific cargoes by molecular motors (Maeder et al., 2014; Franker and Hoogenraad, 2013). Molecular motors play central roles in intracellular transport, and have diversified through evolution (Vale, 2003). However, the diversity of molecular motors is not sufficient to explain transport specificity, as various cargoes often share the same motor while being transported to distinct locations. For example, cytoplasmic dynein is the major microtubule minus-end motor and transports a variety of cargoes including the Golgi apparatus, endosomes and RNAs (Kardon and Vale, 2009). Evidence suggests that the fate of cargo is determined not only by cargo-motor recognition, which occurs upon cargo loading, but also at the cargo destination site. For instance, in axonal transport some cargoes, such as dense core vesicles and synaptic vesicles, are inefficiently captured at synaptic boutons and circulate within the axon (Wong et al., 2012), whereas others, such as mitochondria, are stably retained at synapses (Kang et al., 2008). Although the precise regulation of cargo transport is important for the functional differentiation of various polarized cells, the underlying molecular mechanisms remain poorly understood.

Cell elongation is a widely observed morphogenetic event that requires the coordinated input of intracellular transport, the cytoskeleton and cell polarity (Hepler et al., 2001; Riquelme, 2013). Drosophila bristles, which are hair-like unicellular structures that function as external sensory organs, are formed by the elongation of trichogen cells, which can grow up to 350 μm in 1 day during the pupal stage (Lees and Picken, 1945; Lees and Waddington, 1942; Tilney and DeRosier, 2005). IkB kinase ε [IKKε; also known as IxB kinase-like 2 (Ik2)] acts at the distal tip of growing bristles and functions as a signaling center to regulate the bidirectional shuttling of Rab11-positive recycling endosomes during bristle elongation (Otani et al., 2011). Rab11-positive vesicles are transported to the distal tip by interacting with cytoplasmic dynein via an adaptor protein Nuf/Rab11FIP3 (Otani et al., 2011; Riggs et al., 2007). At the distal tip, IKKε phosphorylates Nuf to inactivate dynein-dependent trafficking, thereby promoting the directional switching of the recycling endosomes (Gould, 2011; Otani et al., 2011). In addition to its role in endosome trafficking, IKKε regulates the organization of both actin and microtubules (Otani et al., 2011; Bitan et al., 2010, 2012). However, how IKKε is localized to the distal tip of growing bristles is unknown.

Spindle-F (Spn-F) is a coiled-coil protein that interacts with IKKε and has been implicated in regulating IKKε polarized activation (Abdu et al., 2006; Dubin-Bar et al., 2008). In oocytes, the intracellular localizations of Spn-F and IKKε depend on each other (Dubin-Bar et al., 2008), and spn-F and ikkε mutants show similar bristle morphology and oocyte polarization phenotypes, suggesting that they function together (Abdu et al., 2006; Koto et al., 2009; Oshima et al., 2006; Otani et al., 2011; Shapiro and Anderson, 2006). Several proteins other than IKKε, including Cut up (Ctp)/dynein light chain (LC8) and Javelin-like (Jvl), are reported to interact with Spn-F (Abdu et al., 2006; Dubin-Bar et al., 2011). It was proposed that Spn-F interacts with cytoplasmic dynein via Ctp...
RESULTS

Spn-F stably localizes to the distal tip of the elongating bristle

To elucidate the relationship between IKKe and Spn-F in bristle elongation, we co-stained developing bristles with anti-Spn-F and anti-phosphorylated IKKe (at serine 175; pIKK\(\epsilon\)) antibodies. Spn-F and pIKK\(\epsilon\) accumulated and colocalized at the tip of growing bristles (Fig. 1A) (Bitan et al., 2010; Otani et al., 2011).

We next examined the dynamics of Spn-F by expressing functional Spn-F::GFP, which accumulated at the distal tip (Fig. 2L) and could rescue the spn-F\(\epsilon\) mutant bristle morphology phenotype (Fig. 3F,N). Fluorescence recovery after photobleaching (FRAP) and inverse-FRAP experiments revealed that the Spn-F::GFP at the distal tip did not turnover within 3 min (Fig. 1B-E; supplementary material Movies 1 and 2), as distinct from GFP::Rab11, which turned over within 30 s (Otani et al., 2011). These results indicate that the distal tip localization of Spn-F is stable.

Spn-F moves along microtubules in Drosophila S2 cells

To study the Spn-F transport mechanism, we expressed Spn-F::GFP in cultured Schneider 2 (S2) cells and observed its motion. In S2 cells, Spn-F::GFP was localized to puncta that moved along microtubules (Fig. 1F,G; supplementary material Movies 3 and 4). This motion still occurred upon treatment with DMSO (Fig. 1B-E; supplementary material Movies 3 and 4), as distinct from GFP::Rab11, which turned over within 30 s (Otani et al., 2011). These results indicate that the distal tip localization of Spn-F is stable.

Dynein and Spn-F are required for the polarized localization of IKKe

We next examined how the distal tip localization of Spn-F and IKKe is regulated in growing bristles. In control bristles, both Spn-F and pIKKe localized to the distal tip (Fig. 2A,D). In ikke\(^{RNAi}\) bristles, a subset of Spn-F localized to the distal tip, although some also accumulated within the shaft (Fig. 2A-C). By contrast, the pIKKe signal did not accumulate at the distal tip in spn-F\(\epsilon\) bristles (Fig. 2D-F). The tip localization was quantitated by measuring the ‘tip index’ (supplementary material Fig. S1A,B), which has a value of 100 when the signals are completely localized to the distal tip, 0 when they are completely located within the cell body and 50 when they are diffuse (supplementary material Fig. S1C). Spn-F distal tip localization was slightly diminished in ikke\(^{RNAi}\) bristles (Fig. 2I), whereas pIKKe tip localization was severely disorganized in spn-F\(\epsilon\) bristles (Fig. 2J).

To monitor IKKe localization, we used IKKe::myc protein because no available anti-IKKe antibody is sensitive enough for this purpose. As IKKe overexpression is toxic to flies and causes changes in bristle cell fate or morphology (Fig. 3L) (Otani et al., 2011), we replaced some of the endogenous IKKe with low levels of epitope-tagged IKKe by coexpressing IKKe::myc with IKKe hairpin RNA, which targets both endogenous and exogenous IKKe. This resulted in normal bristle morphology (supplementary material Fig. S1D), and IKKe::myc protein accumulated at the distal tip in control bristles (Fig. 2G) but not in spn-F\(\epsilon\) bristles (Fig. 2H,K), indicating that Spn-F is required for IKKe localization to the distal tip in growing bristles.

We next focused on the microtubule minus-end motor cytoplasmic dynein, as the minus-ends of stable microtubules are oriented toward the distal tip (Bitan et al., 2010, 2012). RNAi of Dhc64C, the Drosophila cytoplasmic dynein heavy chain (Gepner et al., 1996; Li et al., 1994; Rasmussen et al., 1994), caused the mislocalization of Spn-F::GFP (Fig. 2L,M,P) and pIKKe (Fig. 2N,O,Q). HA-tagged dynein heavy chain (HA::DHC) localized to the distal tip in both control and ikke\(^{RNAi}\) bristles (Fig. 2R-T). These results suggested that cytoplasmic dynein is required for Spn-F tip localization, and that Spn-F is in turn required for IKKe tip localization.

ikke is epistatic to spn-F

To investigate the relationship between Spn-F and IKKe, we first examined their physical interaction. Spn-F and IKKe are reported to interact with each other (Dubin-Bar et al., 2008), which was confirmed by immunoprecipitation experiments in S2 cells. Overexpressed (supplementary material Fig. S2A) or endogenous (supplementary material Fig. S2B) IKKe co-precipitated with overexpressed Spn-F. Moreover, endogenous Spn-F and IKKe were co-immunoprecipitated from ovary lysates, indicating that they form a complex in vivo (Fig. 3A; supplementary material Fig. S2C). These results demonstrated that Spn-F and IKKe interact with each other.

We next examined the genetic interactions between spn-F and ikke in bristle morphology. Wild-type bristles have a thin, elongated, tapered morphology (Fig. 3B), whereas in spn-F\(\epsilon\) or ikke\(^{RNAi}\) flies the bristles are short, branched and have a characteristic swollen region (Fig. 3C,D) (Abdu et al., 2006; Oshima et al., 2006; Shapiro and Anderson, 2006). We generated ikke\(^{RNAi}\) spn-F\(\epsilon\) double-mutant bristles and found no additive effects as compared with the single mutants (Fig. 3E). This was confirmed by measuring the lengths of the scutellar bristles in each genotype (Fig. 3M). The lack of additive effects in ikke\(^{RNAi}\) spn-F\(\epsilon\) double-mutant bristles suggested that spn-F and ikke function in the same genetic pathway.

To elucidate the relationship between spn-F and ikke, we performed a genetic epistasis analysis. The low-level expression of IKKe::myc in wild-type flies reduced the number of sensory organs (Fig. 3L), which is consistent with a previous report suggesting a role for IKKe in sensory organ precursor development (Kuranaga et al., 2006), although the morphology of the remaining bristles was normal, indicating that IKKe::myc does not affect bristle morphogenesis once the cell fate has been determined (Fig. 3L). IKKe::myc rescued the defects in ikke mutant bristles (Fig. 3K,O). Furthermore, IKKe::myc suppressed the bristle...
morphology defects in \textit{spn-F1} mutants (Fig. 3C,G,N). In some animals, bristles with a hooked morphology were occasionally observed (supplementary material Fig. S2D), suggesting that IKK\textepsilon requires Spn-F to fully exert its function. By contrast, when Spn-F was overexpressed, the bristle morphology was largely normal, with only the occasional appearance of hooked bristles (Fig. 3H). Spn-F overexpression rescued the defects in \textit{spn-F1} bristles (Fig. 3F,N), but failed to suppress the bristle morphology defects in \textit{ikk}\textepsilon mutants (Fig. 3I,J,O; supplementary material Fig. S2E,F). Taken together, these results demonstrated that \textit{ikk}\textepsilon is epistatic to \textit{spn-F}.

\textbf{Spn-F interacts with IKK\textepsilon, Ctp and DHC through distinct regions}

The above results suggested that Spn-F acts upstream of IKK\textepsilon to regulate IKK\textepsilon distal tip localization. To elucidate the molecular mechanisms of this IKK\textepsilon localization, we performed a structure-
Fig. 2. See next page for legend.
Fig. 2. Spn-F and cytoplasmic dynein are required for the polarized localization of IKKε. (A-F) Spn-F (A-C, magenta) and pIKKe (D-F, magenta) localization in control, ikkεRNAi and spn-F mutant bristles at 33 h APF. Spn-F localizes to the tip of control (A) and ikkεRNAi (B) bristles (arrowhead). Some aggregation of Spn-F in the bristle shaft is observed in ikkεRNAi bristles. (C) The Spn-F signal is greatly reduced in spn-F bristles. The remaining signals were background, as spn-F is a null allele. (D) pIKKe localizes to the tip of control bristles (arrowhead). (E) The pIKKe signal is lost in ikkεRNAi bristles. (F) pIKKe tip localization is lost in spn-F2 bristles. (G,H) IKKε::mCh (magenta) localizes to the distal tip (arrowhead) at 40 h APF in ikkεRNAi IKKε::mCh (G) but not in spn-F (H) bristles. Bristle morphology is shown by F-actin (green) in A-H. (I-K) Quantification of Spn-F (I), pIKKe (J) and IKKε::mCh (K) tip localization. (L-O) Spn-F::GFP (L and M) and pIKKe::N (N) localization in control and Dhc64C RNAi (Dhc64CGRNAi) bristles. Spn-F::GFP and pIKKe::N localize to the tip (arrowheads) of control bristles (36 h APF in L, 40 h APF in N) but not in Dhc64C RNAi bristles (20 h APF in M, 24 h APF in O, animals raised at 32°C). Dotted lines (M,O) outline the cell. (P,Q) Quantification of the tip localization of SpnF::GFP (P) and pIKKe::N (Q). (R,S) HA::DHC localizes to the tip (arrowheads) of control (R) and ikkεRNAi (S) bristles at 33 h APF. (T) Quantification of HA::DHC tip localization. Error bars indicate s.d.; n=2-4 bristles analyzed. *P<0.05, **P<0.005, ***P<0.0005; n.s., not significant. See also supplementary material Fig. S1 and S2. Scale bars: 10 µm.

function analysis of Spn-F. The Spn-F protein has two coiled-coil regions (CCs), which we designated CC1 and CC2 (Fig. 4A). We generated various deletion mutants of Spn-F (Fig. 4A) and tested their ability to interact with IKKε. We performed pull-down experiments using ovary lysates. DHC interacted with the C-terminus of Spn-F (21 amino acids 212-252 as the IKKε-interacting region (Fig. 4C,D), whereas the Ctp-interacting region was mapped to amino acids 231-274 ( supplementary material Fig. S3D). These results suggested that IKKε and Ctp interact with distinct regions of Spn-F.

To test whether Ctp mediates the interaction between Spn-F and cytoplasmic dynein, we analyzed the interaction between Spn-F and dynein heavy chain (DHC). Immunoprecipitation using ovary lysates revealed that endogenous DHC co-precipitated with Spn-F (Fig. 4E,F), suggesting that Spn-F forms a complex with cytoplasmic dynein. To determine the cytoplasmic dynein-binding region of Spn-F, we prepared GST-fusion Spn-F fragments (Fig. 4G) and performed pull-down experiments using ovary lysates. DHC interacted with the C-terminus of Spn-F (C2 region) but not with the Ctp-interacting (C1) region (Fig. 4H), indicating that Ctp binding is dispensable for Spn-F to interact with cytoplasmic dynein. These results demonstrate that Spn-F interacts with IKKε, Ctp and cytoplasmic dynein through distinct regions (Fig. 4I).
Fig. 3. \textit{ikke} is epistatic to \textit{spn-F}. (A) Co-immunoprecipitation of IKKe with Spn-F. The Spn-F signal in the ovary lysate is too weak to detect. IP, immunoprecipitation; WB, western blot. (B-L) SEM images of scutellar bristles of the indicated genotypes. (B’-L’) Magnified images of bristle morphology. (B) Control bristles. \textit{spn-F1} (C), \textit{ikk}\textsubscript{66} RNAi (D) and \textit{ikk}\textsubscript{66} RNAi \textit{spn-F1} (E) bristles are short and branched. (F) \textit{Spn-F} overexpression rescues the \textit{spn-F1} mutant bristle phenotype. (G) IKKe overexpression reduces the number of sensory organs and suppresses the \textit{spn-F1} bristle phenotype. (H) \textit{Spn-F} overexpression does not severely affect bristle morphology. (I) \textit{ikk}\textsubscript{66} mutant bristles are short and branched (arrowheads). (J) \textit{Spn-F} overexpression does not suppress the \textit{ikk}\textsubscript{66} bristle phenotype (arrowhead). (K) IKKe overexpression rescues the \textit{ikk}\textsubscript{66} bristle phenotype (arrowheads). (L) IKKe overexpression reduces the number of sensory organs, although the remaining bristles are morphologically normal. (M-O) Quantification of bristle morphology. Length of scutellar bristles was measured. Error bars indicate s.d.; \(n\geq 3\) bristles analyzed. *\(P<0.05\). See also supplementary material Fig. S2. Scale bar: 100 µm.
**Fig. 4.** See next page for legend.
in S2 cells (Fig. 7A, B), and Jvl::GFP formed relatively large puncta at the center of the cell where it colocalized with endogenous Spn-F (Fig. 7B). Coexpression of the two proteins at low levels resulted in their partial colocalization at cytoplasmic punctate structures (Fig. 7C), whereas co-overexpression resulted in the formation of filamentous bundles, where Spn-F::mCh and Jvl::GFP colocalized (Fig. 7D). These structures colocalized with α-tubulin::GFP, suggesting that they were microtubule bundles, consistent with previous observations (Dubin-Bar et al., 2011) (Fig. 7E). The ability of Spn-F to form oligomers (Fig. 7F) suggests that Spn-F and Jvl could form higher-order complexes, although we cannot completely rule out the possibility that the large Spn-F/Jvl-containing puncta observed in S2 cells are aggregates formed by overexpression.

Time-lapse imaging revealed that the Jvl::GFP puncta were highly dynamic (Fig. 7G; supplementary material Movie 9). This mobility was abolished by Colchicine (Fig. 7I; supplementary material Movie 11) but not by Latrunculin A (Fig. 7H; supplementary material Movie 10), suggesting that Jvl::GFP mobility depended on microtubules. In contrast to the majority of Jvl::GFP puncta, the large Jvl::GFP-positive puncta that colocalized with endogenous Spn-F (Fig. 7B) were immobile (Fig. 7G). To examine how the interaction of Spn-F and Jvl affected their mobility, we coexpressed Spn-F::mCh and Jvl::GFP and performed time-lapse imaging (supplementary material Movie 12). Intriguingly, Spn-F::mCh/Jvl::GFP double-positive puncta were immobile (Fig. 7J, white arrow), whereas Spn-F::mCh or Jvl::GFP single-positive puncta within the same cell were able to move (Fig. 7J, magenta arrow for Spn-F::mCh). Overexpression of Spn-F::mCh and Jvl::GFP completely immobilized the two molecules (Fig. 7K; supplementary material Movie 13). These results suggested that Spn-F and Jvl immobilize each other in S2 cells.

**Jvl interacts with microtubules through its C-terminal region**

To elucidate how its binding to microtubules is regulated, we performed a structure-function analysis of Jvl (Fig. 7L). Full-length Jvl localized to punctate structures that were located along microtubules (Fig. 7M). The N-terminal half of Jvl (Jvl[N]) localized to punctate structures, but failed to colocalize with microtubules (Fig. 7N). By contrast, the C-terminal half of Jvl (Jvl[C]) uniformly decorated microtubules (Fig. 7O). These results suggested that Jvl interacts with microtubules through its C-terminal region.

**DISCUSSION**

**The distal tip acts as a sorting station for cytoplasmic dynein-dependent cargoes**

Here we demonstrated that the bristle tip is a sorting station for cytoplasmic dynein-dependent cargoes. The IKKe–Spn-F complex, which acts as the signaling center in bristle cell elongation, localizes to the distal tip by dynein-dependent polarized transport and Jvl-dependent selective retention (Fig. 8A). By contrast, Rab11-positive recycling endosomes undergo both dynein-dependent distal transport and proximal transport, which is probably mediated by kinesins (Fig. 8B) (Gould, 2011; Otani et al., 2011).

The distinct transport characteristics at the distal tip are specified by the nature of the adaptor proteins. IKKe is transported to the distal tip by dynein via the adaptor protein Spn-F, and the IKKe–Spn-F complex is stably retained at the distal tip by Jvl, a Spn-F-interacting protein. By contrast, Rab11-positive recycling endosomes are transported to the distal tip by dynein via the adaptor protein Nuf, where it is phosphorylated by IKKe (Otani et al., 2011). This phosphorylation inactivates the dynein-dependent transport of Rab11-positive recycling endosomes, thereby promoting their transport back to the cell body (Otani et al., 2011). Thus, the IKKe–Spn-F complex stably localizes to the distal tip by polarized transport followed by selective retention, whereas Rab11-positive recycling endosomes bidirectionally shuttle by polarized transport and motor switching. The pivotal step in this sorting decision is the specific recognition of the cargo adaptor proteins (Spn-F and Nuf) by their regulatory proteins (Jvl and IKKe) at the distal tip. These results support the emerging concept that cargo adaptor proteins are not merely physical linkers between cargoes and motors, but act as regulatory hubs where various signals converge (Fu and Holzbaur, 2014).

**Spn-F regulates the localization and function of IKKe**

Our results suggest that Spn-F functions as a cargo adaptor for IKKe and cytoplasmic dynein. Structure-function analysis of
Spn-F demonstrated that its dynein-binding region is required for localizing IKKε to the distal tip and for bristle morphogenesis. In contrast to the dynein-binding-deficient Spn-F mutant, which partially suppressed the spn-F mutant bristle morphology phenotype, a mutant lacking the IKKε-binding region completely failed to rescue, indicating that, in addition to its function as a cargo adaptor, Spn-F has a role in regulating IKKε activity. This role could involve regulating IKKε kinase activity or protein stability, or in scaffolding the components of the IKKε signaling pathway. IKKε overexpression could partially suppress the spn-F mutant bristle morphology phenotype despite IKKε delocalization from the distal tip, suggesting that increasing the dosage of IKKε can compensate for the loss of Spn-F to some extent. It is likely that the delocalized IKKε can phosphorylate...
Conclusions

In summary, we have demonstrated that the signaling center for bristle elongation is localized to the distal tip by polarized transport and selective retention mechanisms. The distal tip of bristles acts as a sorting center for cytoplasmic dynein cargoes, where regulatory proteins recognize cargo adaptor proteins and determine whether cargo is retained or sent back to the cell body. These findings support the idea that cargo adaptor proteins act as regulatory hubs where various signals converge. It would be interesting to test whether the differential regulation of cargo-motor interactions contributes to the formation of signaling centers during the morphogenesis of mammalian cells of complex shape, such as neurons and podocytes.

MATERIALS AND METHODS

Molecular biology

*spin-F* cDNA was cloned by RT-PCR from S2 cells. *ctp* cDNA was from the Drosophila Genomics Resource Center (clone LD24056). *jvl* cDNA (Dubin-Bar et al., 2011) and *IKKε[WT]* and *IKKε[K41A]* constructs were characterized previously (Oshima et al., 2006). The *Spn-F* (ΔN, ΔC2, N, M, C, N1, N2, C1, C2) and *Jvl* (N, C) deletion mutants were generated by PCR; ΔC1 was generated by inserting the corresponding annealed synthetic oligonucleotide into the *BstEII/BstXI* sites of *spin-F* (Hokkaido System Science); Δ191, Δ212, Δ232, Δ253 were generated by synthesizing the corresponding gene fragments and subcloning them into the *BstEII/BstXI* sites of *spin-F* (GenScript). Fusion constructs were generated by subcloning...
Fig. 7. See next page for legend.
Fig. 7. Jvl and Spn-F immobilize each other in S2 cells. (A,B) Localization of (A) SpnF::mCh (magenta) and (B) Jvl::GFP (green) to punctate cytoplasmic structures in S2 cells. The large puncta of Jvl::GFP (arrow) colocalize with endogenous Spn-F (magenta). (C) SpnF::mCh (magenta) and Jvl::GFP (green) partially colocalize. (D) Overexpressed SpnF::mCh (magenta) and Jvl::GFP (green) colocalize and form filamentous structures. (E) α-tubulin::GFP (green) colocalizes with Jvl::mCh (magenta) and Spn-F::HA (blue). (F) Spn-F forms oligomers in S2 cells. (G-I) Mobility of Jvl::GFP depends on microtubules. S2 cells expressing Jvl::GFP were treated with DMSO (G), 1 µM Latrunculin A (H) or 10 µM Colchicine (I). (J) At moderate expression levels, SpnF::mCh/Jvl::GFP double-positive particles (white arrows) are immobile, whereas Jvl::GFP or SpnF::mCh (magenta arrow) single-positive particles can move. (K) Upon overexpression, Jvl::GFP/SpnF::mCh double-positive structures are immobile. Red, 0 s; green, 3 s; blue, 6 s. (L) The Jvl constructs used in M-O. (M-O) Microtubule (magenta) localization of Jvl deletion mutants (green) in S2 cells. Jvl[FL]:GFP puncta localized along microtubules (M, arrows), whereas Jvl[N]:GFP puncta did not overlap with microtubules (N). Jvl[C]:GFP uniformly decorated microtubules (O). See also supplementary material Movies 9-13. Scale bars: 10 µm.

Drosophila stocks

The following Drosophila strains were used: y w 67C21 as a control; spn-FΔ (Abdu et al., 2006), ikke△ (Oshima et al., 2006), ikkeG354 (Oshima et al., 2006), ikkeN50 (Oshima et al., 2006) and jvlΔ (Dubin-Bar et al., 2011) were described previously; ikkeG354 was provided by Kathryn Anderson (Shapiro et al., 2006); Dhc64CΔ wN50 (P1 GD12258v2 8054) was provided by the Vienna Drosophila RNAi Center; and UAS-HA::Dhc64C was provided by Tom Hays (Silvanovich et al., 2003). Drosophila were raised at 25°C with the following exceptions: 16-20°C for IKKε overexpression by the Sca-Gal4 tub-Ga180p driver, and 30-32°C for Dhc64C RNAi by the neu-PGal4-72::GAL4 driver. Transgenic flies were generated by standard P-element-mediated transgenesis, and overexpression was performed using the Gal4-UAS system (Brand and Perrimon, 1993). Sca-Gal4 (de Celis et al., 1999), neu-PGal4-72 (a kind gift from François Schweisguth, Institut Pasteur, Paris, France) (Bellaiche et al., 2001), neu-PGal4-72 tub-Gal80p (provided by Adrian Moore, RIKEN-BSI, Japan) and Sca-Gal4 tub-Gal80p (generated by recombination) were used for overexpression; and tub-Gal80p (McGuire et al., 2003) was from the Bloomington Stock Center. ikke mutant clones were generated by the FLP-FRT system (Xu and Rubin, 1993) using Ubx-flp (a kind gift of Jürgen Knoblich) (Emery et al., 2005), and transgenes were expressed in mutant clones by the mosaic analysis with a repressible cell marker (MARMC) system (Lee and Luo, 1999). See supplementary material Table S1 for the genotypes used in each experiment.

Antibodies

Guinea pig and rabbit anti-Spn-F N-terminus antibodies were generated by injecting purified GST-Spn-F-N (amino acids 1-190) into guinea pigs and rabbits. The immunization and affinity purification by antigen-conjugated column were performed by MBL. The mouse anti-Spn-F antibody (8C10) (Abdu et al., 2006), mouse anti-IKKε antibody (clone 80) (Oshima et al., 2006) and affinity-purified rabbit anti-pIKKε antibody (S175) (Otani et al., 2011) were described previously. Mouse antidynein heavy chain monoclonal antibody (clone 2C11-2) was from the Developmental Studies Hybridoma Bank (Sharp et al., 2000). See supplementary material Table S2 for a full description of the antibodies used.

Cell culture and immunofluorescence

Drosophila S2 cells were cultured in Schneider’s Insect Medium (Gibco) supplemented with 10% FCS and antibiotics at 25°C (Schneider, 1972). PUAST vectors with actin5Ce-Gal4 drivers were cotransfected using Effectene (Qiagen) according to the manufacturer’s instructions, and harvested 36-48 h after transfection. For immunofluorescence or time-lapse imaging, cells were replated on coverslips or glass-bottom dishes coated with Concanavalin A (Wako) and were allowed to spread for 1-2 h (Rogers et al., 2002). For drug treatments, cells were treated with 1 µM Latrunculin A (Wako) or 10 µM Colchicine (Wako) for 1 h before imaging. For immunofluorescence, cells were fixed in 4% paraformaldehyde in PBS–0.1% Triton X-100 in PBS (PBS-T) for 15 min, and blocked with 5% skimmed milk in Tris-buffered saline (TBS). Primary and secondary antibodies were diluted in the blocking solution. After each antibody incubation, the coverslips were washed three times with PBS–T. The cells were mounted in Vectashield mounting medium (Vector Labs).

Fig. 8. Model for bristle tip IKKε–Spn-F transport and retention. (A) Transport and retention of the IKKε–Spn-F complex. The IKKε–Spn-F complex is transported to the distal tip by cytoplasmic dynein. Jvl is independently transported to the distal tip and interacts with Spn-F to retain the IKKε–Spn-F complex there. (B) The shuttling movement of recycling endosomes. Rab11-positive recycling endosomes are transported by cytoplasmic dynein to the distal tip. IKKε phosphorylates Nuf, a Rab11-dynein adaptor protein, and promotes motor switching. Specific pairings between cargo adaptors (Spn-F, Nuf) and regulatory molecules (Jvl, IKKε) determine cargo fate.
Immunohistochemistry

Pupae were fixed as described previously (Otani et al., 2011). Blocking was performed in 0.1% BSA, 0.2% Triton X-100 and 0.2% Tween 20 in PBS overnight at 4°C. Primary and secondary antibodies were diluted in the blocking solution and incubated with the sample overnight with gentle agitation at 4°C. Samples were washed with PBS-T three times after antibody incubation steps. The thorax pieces were mounted dorsal side up on glass slides in Vectashield mounting medium and covered with a coverslip; a second coverslip was used as a spacer.

Confocal microscopy

Confocal microscopy was performed on an FV1000-BX61 laser-scanning confocal microscope using an UPlanSapo 60×/NA 1.35 objective (all Olympus). Movies were captured using the FV1000-IX81 microscope using a PlanApo N 60×/NA 1.42 objective (all Olympus). Macrochaetes were imaged for all experiments. z-stack image generation and brightness and contrast adjustment were performed using ImageJ (NIH) without any nonlinear adjustments. Gaussian filter was applied to generate still images from time-lapse imaging of S2 cells.

The ‘tip index’ was determined as follows (see also supplementary material Fig. S1). A line scan was performed from the base of the bristle to the distal tip to obtain a plot profile using ImageJ. Subsequent analyses were performed using Excel (Microsoft). The maximum intensity (100% intensity) and bristle length (Position(Max)) were determined from the line scan, and pixels that exceeded 50% intensity were identified. The tip index was defined as the relative position of the pixels that exceeded 50% intensity along the proximal-distal axis of the bristle; the full bristle length was defined as 100. Statistical analyses (Student’s t-test) were performed using Excel.

Scanning electron microscopy (SEM)

Adult flies were anesthetized by CO2 and the legs and wings were removed by fine forceps. The dissected flies were mounted dorsal side up, sputter-coated with platinum (JFC-1600; JEOL) or osmium (Neoc-STB; Meiwafosis) and viewed with a scanning electron microscope (JSM-5600-LV; JEOL) at low vacuum (30 Pa) using an acceleration voltage of 10 kV. Scutellar bristles were imaged for all experiments. The scutellar bristle length was measured by ImageJ, and statistical analyses (Student’s t-test) were performed using Excel.

Biochemistry

Transfected S2 cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM DTT). To generate ovary extracts, adult female flies were cultured on yeast for 3 days. The flies were anesthetized by CO2 and the ovaries were dissected under PBS. The dissected ovaries from 40 control females or 100 spin-F mutant females were then homogenized in 1 ml lysis buffer; more spin-F mutant ovaries were used because they were underdeveloped due to oogenesis defects (Abdu et al., 2006). The lysates were incubated for 30 min at 4°C, then cleared by centrifugation at 20,000g. Supernatant and proteins were expressed at 20°C for 16-20 h for GST-N, or at 37°C for GST-C, GST-C1 and GST-C2.

For GST pull-down assays, GST, GST-N, GST-C, GST-C1 and GST-C2 were expressed in BL21 (DE3) pLysS E. coli cells (Novagen). Protein expression was induced by adding 0.1 mM IPTG to the bacterial cultures, and proteins were expressed at 20°C for 16-20 h for GST-N, or at 37°C for 3 h for GST-C, GST-C1, GST-C2 and GST-C2. Recombinant protein purification was as described previously (Otani et al., 2011). For the pull-down assays, 20 µg GST-fusion protein was added to ovary lysate, and the mixture was incubated overnight with rotation at 4°C. Glutathione-Sepharose 4B beads (GE Healthcare) were then added, and the samples were incubated with rotation for 2 h at 4°C. The beads were rapidly washed three times with lysis buffer, and the complexes were eluted by boiling in 2× Laemmli sample buffer supplemented with 10% β-mercaptoethanol. To detect interactions with DHC, it is essential that the washes are performed rapidly.

SDS-PAGE was performed by standard methods using 15% (to detect Ctp-myc) or 5-20% SuperSep Ace polyacrylamide gels (Wako). Western blotting was performed as described previously (Otani et al., 2011).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

T.O., K.O. and S.H. designed the study. T.O. performed the experiments with the assistance of A.K.K.O., M.T. and U.A. contributed reagents. T.O. and S.H. wrote the paper, and all authors edited the manuscript.

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Supplementary material

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