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Rab11 is required for cell adhesion, maintenance of cell shape and actin-cytoskeleton organization during *Drosophila* wing development

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ABSTRACT Intracellular protein trafficking is a key factor in maintaining epithelial cell adhesion and cell shape. Small monomeric Rab GTPases are key players involved in intracellular membrane transport. Rab11, a subfamily of the *Ypt/Rab* gene family of ubiquitously expressed GTPases, is associated with recycling endosomes, and acts as a master molecule in regulating vesicular trafficking. Wing epithelium of *Drosophila* has been chosen to address the involvement of Rab11 in trafficking of a cell adhesion molecule, the βPS integrin. Here, we show that Rab11 immunocolocalizes with trans-Golgi network and it is enriched in the centrosomal/recycling endosomal area labeled by γ-Tubulin. Furthermore, Rab11 is required for transcytic and exocytic trafficking of βPS integrin; alterations of Rab11 function by different genetic procedures in wings results in the formation of blisters. We show altered activity of Rab11 affects cell adhesion, cell shape and organization in the actin-cytoskeleton during wing morphogenesis. Finally, using a genetic approach, we demonstrate that Rab11 interacts with the βPS integrin. Collectively, our data suggest that Rab11 regulates cell adhesion, maintenance of cell shape and actin-cytoskeleton organization during *Drosophila* wing development.

KEY WORDS: *Rab11, vesicular trafficking, recycling endosome, βPS integrin, cell shape*

Introduction

Intracellular vesicle trafficking plays an important role in a wide range of biological processes including pattern formation, establishment of cell polarity, maintenance of cell shape and several developmental signalling pathways as well as uptake of nutrients and particles. Traditionally, most of the protein/vesicle trafficking studies have been carried out in yeast cells or in mammalian cell lines. Recent analysis of protein trafficking in whole organisms revealed that vesicle trafficking plays exciting roles in the development of multicellular organisms. Trafficking of different cell adhesion molecules regulates epithelial polarity, cell shape changes and cell migration. Developmental signalling cascades are also regulated by the endocytic and exocytic trafficking of receptors and their ligands (Gonzalez-Gaitan, 2003; Piddini and Vincent, 2003).

Membrane transport in eukaryotic cells is a complex process regulated by a large and diverse array of proteins. A large group of monomeric small GTPases: the Rabs, comprised of a small family of ubiquitously expressed proteins are essential components of the membrane-transport pathway. The Rabs are implicated in vesicle formation, loading, transport along cytoskeleton elements and finally docking and fusion with the target membranes (Zerial and McBride, 2001). Each Rab protein is localized to the cytoplasmic surface of a distinct membrane bound organelle (Ferro-Novick and Novick, 1993; Novick and Zerial 1997; Pfeffer, 1994; Takai *et al.*, 1992; Zerial and Stenmark, 1993) and appears to control a specific membrane transport pathway. In the steady state, Rab proteins accumulate at their target compart-

Abbreviations used in this paper: AJ, adherens junctions; DABCO, 1,4 diazobicyclo octane; FLP, flippase, FRT, FLPase recognition target; Gal4, galactose4; GDI, guanine dissociation inhibitor; GFP, green fluorescent protein; LE, leading edge cell; PBS, phosphate-buffered saline, PFA, paraformaldehyde; RE, recycling endosome; SOP, sensory organ precursor; TGN, trans-Golgi network; UAS, upstream activating sequence.

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ment and thereby have been used as markers for different organelles (Bucci *et al.*, 1992; Chavrier *et al.*, 1990; Chavrier *et al.*, 1991; Ullrich *et al.*, 1996). Only a minor fraction of each Rab protein is localized in the cytosol where it remains complexed with a protein called guanine dissociation inhibitor (GDI) (Garrett *et al.*, 1993; Regazzi *et al.*, 1992; Sasaki *et al.*, 1990; Sasaki *et al.*, 1991; Soldati *et al.*, 1993; Ullrich *et al.*, 1993). As with other GTPases, Rab proteins also shuttle between two activity states: an inactive GDP-bound state and an active GTP-bound state. In the GTP-bound state, Rabs are associated with membranes and are attached to the cytoplasmic surface of compartments (Pfeffer and Aivazian, 2004). Mutations in Rab GTPases affect cell growth, motility and other biological processes.

Rab11, a subfamily of Rab GTPases, is a well known marker for recycling endosomes (REs), localizes to the pericentriolar recycling of endosomal compartment, the trans-Golgi network (TGN) and post-Golgi vesicles (Chen *et al.*, 1998; Deretic, 1997; Ullrich *et al.*, 1996) and plays a key role in transporting both the endocytosed proteins and some newly synthesized proteins through REs to the plasma membrane (Ang *et al.*, 2004; Chen *et al.*, 1998; Lock and Stow, 2005; Ren *et al.*, 1998; Satoh *et al.*, 2005; Ullrich *et al.*, 1996).

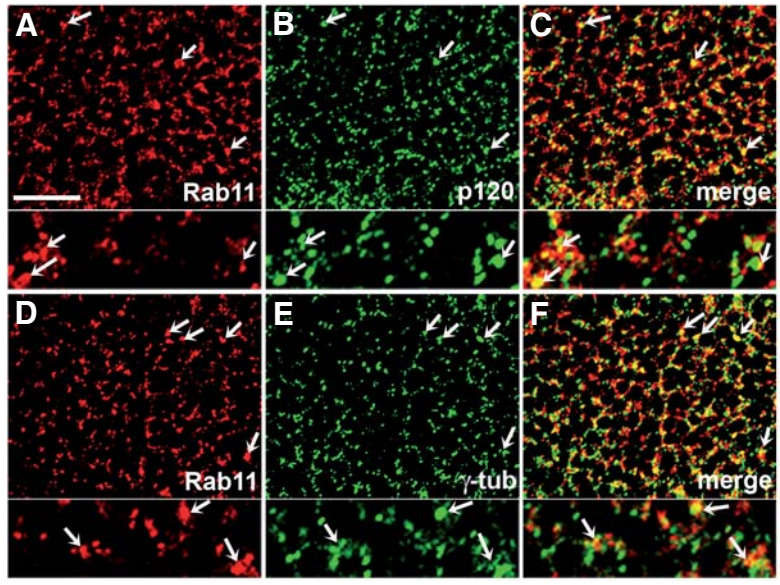


Fig. 1. Rab11 immunolocalization in wild type wing imaginal disc cells. *Rab11* **(A,D)** *forms small vesicular/punctate structures throughout the cytoplasm. These punctate structures co-localize (arrows in* **(C)***) with TGN marker p120 (arrows in* **(B)***) and are enriched in the centrosomes (arrows in* **(F)***) as marked with γ-tubulin (arrows in* **(E)***). Scale bar, 10 μm.*

The developing wing of *Drosophila* has been extensively used as a model system for studying cell shape changes associated with rearrangements of epithelial cells during morphogenesis in an intact animal. Mutations or other manipulations can easily be done in wing of *Drosophila* because it has little effect on overall viability of the organisms. The large size of wing facilitates the process of genetic screening and scoring of different mutant phenotypes. Wing morphogenesis is a relatively simple process involving the conversion of a single layered columnar epithelium to a flattened bilayer where the basal surfaces of dorsal and ventral epithelia are in close contact. Basically, wing development starts from about 30 precursor cells in the embryo which after several rounds of cell divisions, invaginate from embryonic ectoderm to form wing imaginal disc. Like other discs, wing imaginal disc of late third instar larva is a flattened, two-sided sac comprising of a columnar cell epithelium and an overlying squamous cell layer, the peripodial epithelium (Held, 2002). Each disc contains about 50,000 cells and gets metamorphosed at pupal stages to adult wing as well as a part of the thorax (Garcia-Bellido and Merrian, 1971).

Integrins, one of the best characterized group of the major cell surface receptors, which are heterodimeric proteins composed of an α- and a β-chain and which mediate cellular connections between the ligands in the extracellular matrix and the actin-cytoskeleton present in animal cells. They are involved in transmission of various signals from the extracellular environment to different signalling pathways in cells (Hynes, 1992; Hynes, 2002). During the last two decades, extensive studies have revealed important roles of integrin during *Drosophila* development and cellular differentiations. For example, integrins are essential for maintaining the close apposition of wing surfaces during wing morphogenesis, attachment of embryonic muscles to epidermis and mediating connections between lateral surfaces of amnioserosa cells and leading edge cells (LE) during embryonic dorsal closure (reviewed by Brown *et al.*, 2000). In the pupal wing, integrins are localized to adhesive contact sites at the basal surface of the epithelia (Fristrom *et al.*, 1993) and elimination of integrins from either wing surfaces in the form of patches of mutant cells or in some cases even partial loss of integrin results in the formation of blister due to the separation of dorsal and ventral wing surfaces in adult (Brower and Jaffe, 1989; Wilcox *et al.*, 1989; Zusman *et al.*, 1990).

In this communication, we have investigated the subcellular localization of Rab11 in protein trafficking pathway in *Drosophila* wing imaginal disc cells. We find that Rab11 is localized both in pericentriolar REs and in the TGN. It has been shown that Rab11 is essential for transcytic and exocytic trafficking of βPS integrin and alterations in Rab11 activity affects its transport. We find that Rab11 plays an important role in maintaining cell shape and actin-cytoskeleton organization during wing morphogenesis, since altered Rab11 activity causes in cell shape changes and disorganization of actin-cytoskeleton. In addition we also show a genetic interaction of Rab11 with βPS integrin.

Results

Rab11 localizes to the TGN and shows pericentriolar localization in Drosophila *wing disc epithelial cells*

Here, to investigate the involvement of Rab11 in membrane/protein transport pathways in wing epithelial cells, it was imperative to determine the localization of Rab11 with respect to the vesicular transport routes. To localize Rab11 at cellular level, a double immunostaining was performed using pericentriolar marker γ-tubulin or trans-Golgi marker p120 with Rab11. Rab11 was found to be scattered throughout the cytoplasm giving a punctate/vesicular pattern of expression (Fig. 1 A,D). When p120 and γ-tubulin were co-immunostained with Rab11, the small punctate structures of Rab11 were found to co-localize (Fig. 1C) signifi-

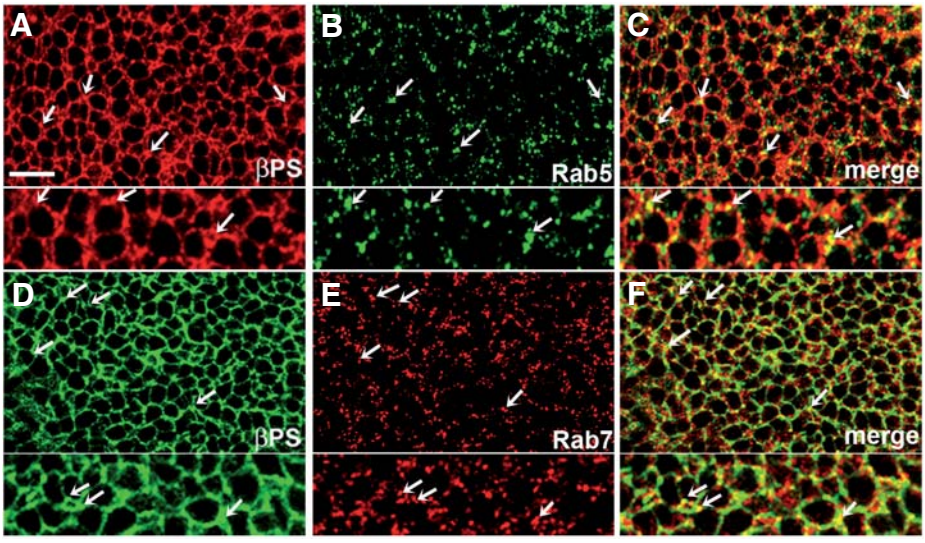


Fig. 2. Endocytic trafficking of βPS integrin in wild type wing imaginal disc cells. *βPS integrin is found in Rab5 and Rab7 endosomes. It is detected as puncta* **(A,D)** *in addition to its membrane localization. Disc showing co-localization (arrows in* **(C)***) of βPS integrin (arrows in* **(A)***) puncta with Rab5 endosomes (arrows in* **(B)***). Disc showing co-localization (arrows in* **(F)***) of βPS integrin puncta (arrows in* **(D)***) with Rab7 (arrows in* **(E)***). Scale bar, 5 μm.*

cantly (42%) with p120 (Fig. 1B) and were enriched (Fig. 1F, 46%) with γ-tubulin, a centrosomal marker."In addition to double immunolocalization of Rab11 with p120, wing imaginal discs expressing Golgi marker, CFP-galactosyl transferase was immunolocalized with Rab11. In this case also most of the Rab11-positive vesicles were found to colocalize with CFP-galactosyl transferase (data not shown). Thus, in wing epithelial cells Rab11 localizes to TGN and shows pericentriolar/recycling endosomal localization. Further, to establish trans-Golgi association of CFP-galactosyl transferase, wing imaginal discs expressing the Golgi marker, CFP-galactosyl–transferase was immunostained with p120 which gives significant colocalization (data not shown). Therefore, CFP-galactosyl transferase can be used as a trans-Golgi marker. On the basis of pericentriolar/recycling endosomal and trans-Golgi association of Rab11, we investigated the transcytic and exocytic trafficking of an important cell adhesion molecule, βPS integrin in wing disc epithelium.

βPS integrin is trafficked through the endocytic pathway to the late endosome in wing disc epithelium

On late third-instar larval wing imaginal discs of *Drosophila, β*PS antibody binds to the basolateral surfaces of both the dorsal and ventral compartment of disc epithelial cells as well as many other tissues (Brower *et al.*, 1984). In higher magnification βPS integrin can be detected at the plasma membrane as well as in discrete puncta (Fig. 2 A,D) suggesting that βPS integrins are internalized. To determine, whether βPS integrins are trafficked through the early endocytic pathway, we examined co-localiztion between Rab5 and βPS integrin. A significant percentage (38%) of βPS integrin puncta co-localized (arrows in Fig. 2C) with Rab5. The localization of βPS integrin to the early endocytic compartments indicates that this molecule is actively endocytosed and may be trafficked to the late endosomes/lysosomes for degradation. As Rab5 is replaced by Rab7 in the late endosomes, we performed colocalization between Rab7 (Fig. 2E) and βPS integrin (Fig. 2D) and a significant percentage (35%) of βPS integrin puncta also co-localized (Fig. 2F) with Rab7. However, in both of the cases (Fig. 2 C,F) the non-colocalizing puncta may be in different compartments. Thus a significant fraction of βPS integrin is trafficked through the endocytic pathway to the late endosomes in wing disc epithelium. In agreement of our finding in endocytic

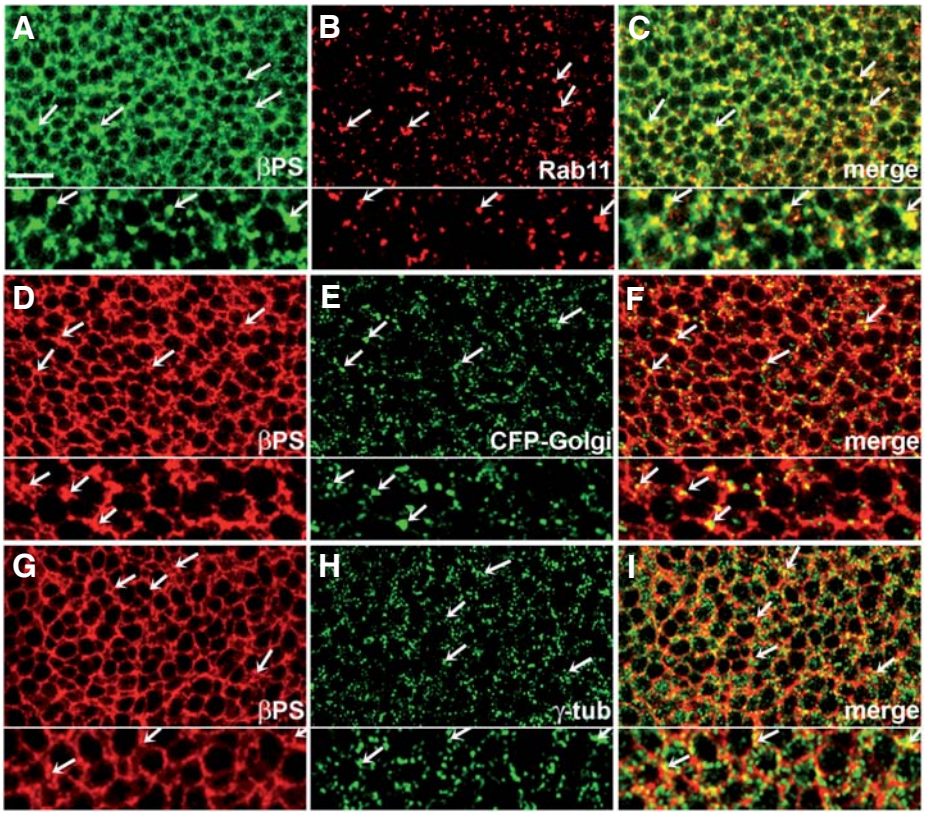


Fig. 3. Transcytic and exocytic trafficking of βPS integrin mediated by Rab11. *βPS integrin is found in Rab11 positive recycling endosomes. It is detected as puncta in addition to its membrane localization* **(A,D,G)**. *Disc showing co-localization (arrows in* **(C)***) of βPS integrin (arrows in* **(A)***) puncta with Rab11 endosomes (arrows in* **(B)***). Disc showing co-localization (arrows in* **(F)***) of βPS integrin puncta* **(D)** *with CFP-stained Golgi (arrows in* **(E)***). Disc showing co-localization (arrows in* **(I)***) of βPS integrin puncta (arrows in* **(G)***) with γ-tubulin (arrows in* **(H)***). Scale bar, 5 μm.*

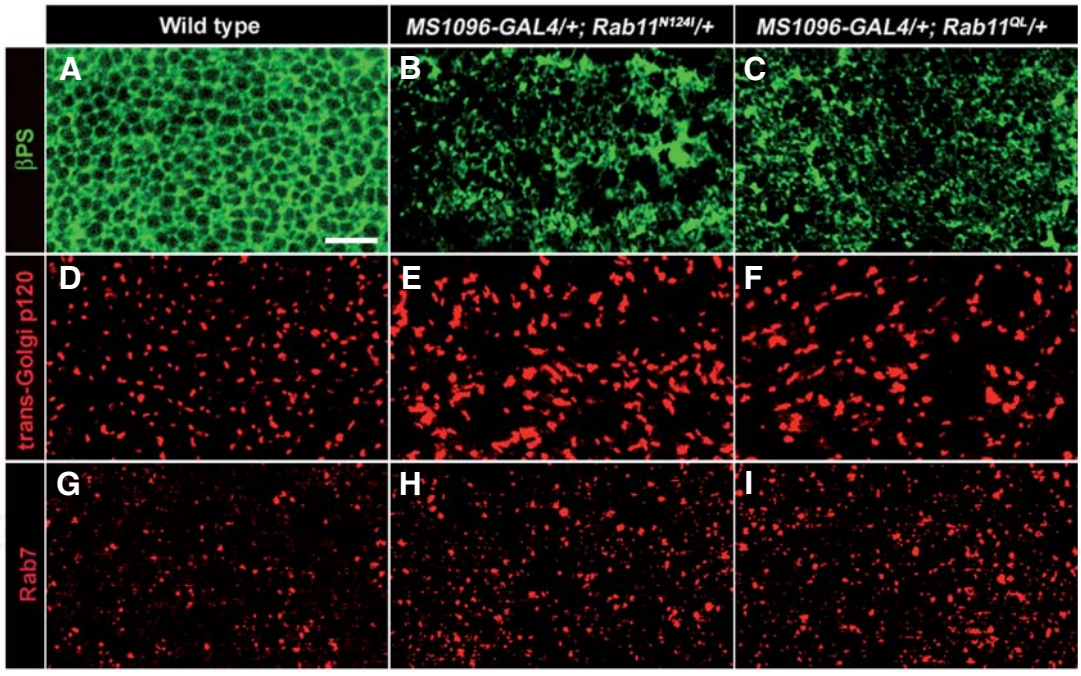


Fig. 4. Dominant-negative or constitutively-active form of Rab11 inhibits βPS integrin transport. *In control wild type wing pouch cells, βPS integrin staining gives punctate pattern of expression in addition to its membrane localization* **(A)**. *In* Rab11^{N124I} **(B)** *or* Rab11^{QL} *expressing* **(C)** *wing pouch cells, βPS integrin transport is inhibited and they are dispersed throughout the cytoplasm instead of being delivered to focal adhesion sites. Enlarged Golgi morphology was observed by staining with trans-Golgi marker p120 in* Rab11^{N124I} **(E)** *or* Rab11^{QL}*-expressing cells* **(F)** *as compared with wild type* **(D)**. *Late endosomes stained with Rab7 were also enlarged in size in over-expressed mutant Rab11 cells as compared with wild type* **(G)**. *Scale bar, 5 μm.*

trafficking of βPS integrin in wing imaginal disc cells a recent article in cell line studies on Rab5 suggested an essential role of this GTPases in the regulation of β1 integrin endocytosis (Pellinen *et al.*, 2006).

Rab11 mediates transcytic and exocytic trafficking of βPS integrin in wing disc epithelium

To test whether βPS integrin is trafficked through Rab11 endosomes in *Drosophila* wing imaginal disc cells, immunostaining were performed for Rab11 (Fig. 3B) and the βPS integrin (Fig. 3A). They show extensive co-localization (75%, arrows in Fig. 3C) indicating a possibility of recycling of endocytosed βPS integrin containing Rab11-positive vesicles and/ or newly synthesized βPS integrin associated with TGN for the exocytic trafficking. To clarify these two possibilities, immunostaining were performed separately for βPS integrin and trans-Golgi marker CFP-stained Golgi or βPS integrin and pericentrosomal/centrosomal marker γ-tubulin (Fig. 3 E,H). In both the cases, a significant percentage of centrosomal marker, γ-tubulin (30%) or trans-Golgi marker, CFP-Golgi positive vesicles (35%) are positive for βPS integrins (arrows in Fig. 3F and I, respectively). Thus, these immunofluorescnce experiments suggest that after endocytosis of βPS integrins, these are recycled back to plasma membrane via Rab11-positive REs and newly synthesized βPS integrins are also trafficked through Rab11-positive vesicles from TGN to plasma membrane. Hence, the subcellular localization of Rab11 is consistent with its transcytic and exocytic function. Further, to test directly whether Rab11 is involved in transcytic and exocytic trafficking of βPS integrin, we investigated βPS integrin transport in dominant-negative or constitutive-active Rab11 wing disc cells. Since null alleles of *Rab11* die as embryos or in early instar larval stages (Jankovics *et al.*, 2001; Dollar *et al.*, 2002), generation of homozygous *Rab11* mutant cells in wings was aimed, but they did not survive (Supplementary Fig. S1), therefore, a dominant-negative (*Rab11^{N124I}*) or a constitutively-active (*Rab11^{QL}*) forms of *Rab11* were expressed in whole wing pouch using *MS1096-GAL4*. Upon expression of dominant-negative or constitutively-active Rab11 proteins, βPS integrins abnormally accumulated/ aggregated throughout the cytoplasm (Fig. 4A compared with 4B and C respectively).The above results indicated that alterations in Rab11 activity cause abnormal accumulation of βPS integrin positive vesicles in the cytoplasm.

Since Rab11 participates in exocytic trafficking and recycling of endocytosed βPS integrin or transcytosis of βPS integrin, it was assumed that abnormal accumulation of βPS integrin positive vesicles could be in TGN or in Rab7 positive late endosomes as alterations in Rab11 activity might cause inhibition of its recycling back to plasma membrane in *Rab11^{N124I}* and *Rab11^{QL}*-expressing wing epithelial cells. So, we assumed that morphology of Golgi and late endosomes might be affected in *Rab11^{N124I}* or constitutively-active *Rab11^{QL}*-expressing wing epithelial cells. Indeed we observed enlarged morphology of both trans-Golgi (Fig. 4D compared with 4 E,F) and late endosomes (Fig. 4G [mean area of puncta 116.11a.u.±22.86] compared with 4H [407.19a.u.±61.49, p>0.10], I [422.61a.u.±74.54, p>0.10]) visualized by p120 and Rab7, respectively, after expression of a dominant-negative or constitutively-active Rab11 proteins, suggesting that inhibition of exocytic transport may impair post-Golgi trafficking and inhibition of recycling may increase trafficking to Rab7 endosomes for βPS integrin degradation. Thus, these findings in agreement with the Rab11 localization studies indicate it's exocytic and transcytic trafficking of βPS integrin.

Over-expression of mutant Rab11 alters wing disc morphology and results in cell shape changes

Since Rab11 mediates transcytic and exocytic trafficking of βPS integrin in wild type third-instar wing disc epithelial cells, the phenotypic consequences were investigated by expressing dominant-negative (*Rab11^{N124I}*) or constitutively-active (*Rab11^{QL}*) or *Rab11dsRNA* using UAS-GAL4 system (Brand and Perrimon,

1993) in wing pouch driven by *MS1096-GAL4* (Fig. 5A). This showed blisters in adult wings with a reduction in wing size (Fig. 5 B,C,D). Presence of blisters suggests loss of integrin function while reduced wing size is due to apoptosis (Fig. 5 F,G,H). Further, to check, whether *Rab11*- induced blister phenotype is not simply a consequence of the loss of cell-cell contacts in dying cells, *DIAP* (an anti-apoptotic gene) was simultaneously expressed with *Rab11* to block apoptosis. An increase in blister size was observed in adult wings (Fig. 5 I,J,K). Quantification of blister is represented in Fig. 5E. One of the hypomorphic allele, *Rab11*^{mo}, in homozygous condition develops upto pharate adult and the escapers showed degenerated eyes (Alone *et al.*, 2005) but they do not show blistering or any other detectable mutant phenotypes in their wings. Since, strong hypomorphic or nearest to null alleles of *Rab11* were lethal so generation of homozygous *Rab11* mutant clones in wing was imperative to confirm whether homozygous *Rab11* mutation causes blisters in adult wings or not. Clones were induced in wing imaginal discs of *yw P{ry*$^{+}$*t7.2hsFLP}22; Rab11*$^{EP(3)3017}$*P{ry[+t7.2]=neoFRT}82B/P{ry[+t7.2]=neoFRT}82B Ubi-GFP*^{nls} 48/72/96 larvae hrs after egg laying and heat shock were given for 45/60/90 min. *Rab11*$^{EP(3)3017}$ mutant homozygous cells did not survive as revealed by the absence of non-GFP cells, whereas their sibling clones with higher levels of GFP did survive normally (Supplementary S1). The phenotypic consequences of adult wings obtained from different hetero-allelic combinations was also carried out using four *Rab11* alleles (*Rab11*^{93Bi}, *Rab11*$^{l(3)j2D1}$, *Rab11*^{mo}, *Rab11*$^{EP(3)3017}$). Only *Rab11*^{93Bi}/*Rab11*$^{l(3)j2D1}$ and *Rab11*^{mo}/*Rab11*^{93Bi} trans-heterozygotes survived up to adults having no observable anomalies in their wings at 25°C and rest *Rab11*$^{EP(3)3017}$/*Rab11*^{mo}, *Rab11*^{93Bi}/*Rab11*$^{EP(3)3017}$ and *Rab11*^{mo}/*Rab11*$^{l(3)j2D1}$ trans-heterozygotes were larval lethal at 25°C and also in 18°C. To better understand the morphology of third instar wing imaginal discs obtained from dominant-negative or constitutively-active *Rab11* driven by *MS1096-GAL4*, phalloidin staining was done which showed an invaginated/folded wing pouch (Fig. 6 B,C) as compared to the wild type (Fig. 6A). For closer examination, scanning electron microscopy was also carried out which supports the altered wing pouch morphology (Fig. 6 E,F compared with 6D). The invaginated wing pouch morphology may be due to over proliferation of cells or could be due to cell shape changes. To check if there is over proliferation of cells, dominant-negative or constitutively-active Rab11-expressing tissues were stained with anti-phosphorylated-histone3 antibody. The overall staining patterns observed on wing imaginal discs from wild type (Fig. 6G) or from discs expressing Rab11^{N124I} or Rab11^{QL} (Fig. 6 H,I), respectively, suggested no over proliferation but a decrease in cell number due to apoptosis (Fig. 5 F,G,H) whereas, in higher magnification of both dominant-negative or constitutively-active Rab11-expressing cells after staining with phalloidin reveals that mutant cells had aberrant shapes. In particular phalloidin stains the cortical actin-filaments associated with the apical adherens junctions, thereby outlining the apical ends of the cells (Condic *et al.*, 1991). Mutant cells for Rab11 appear to be apically widened in comparison to the wild type cells (Supplementary S2). Changes in cell shape might cause an increase in cell surface resulting folds in the wing pouch. Staining on tissues expressing *Rab11*^{N124I} or *Rab11*^{QL} using an apico-lateral marker, anti-phosphoty-

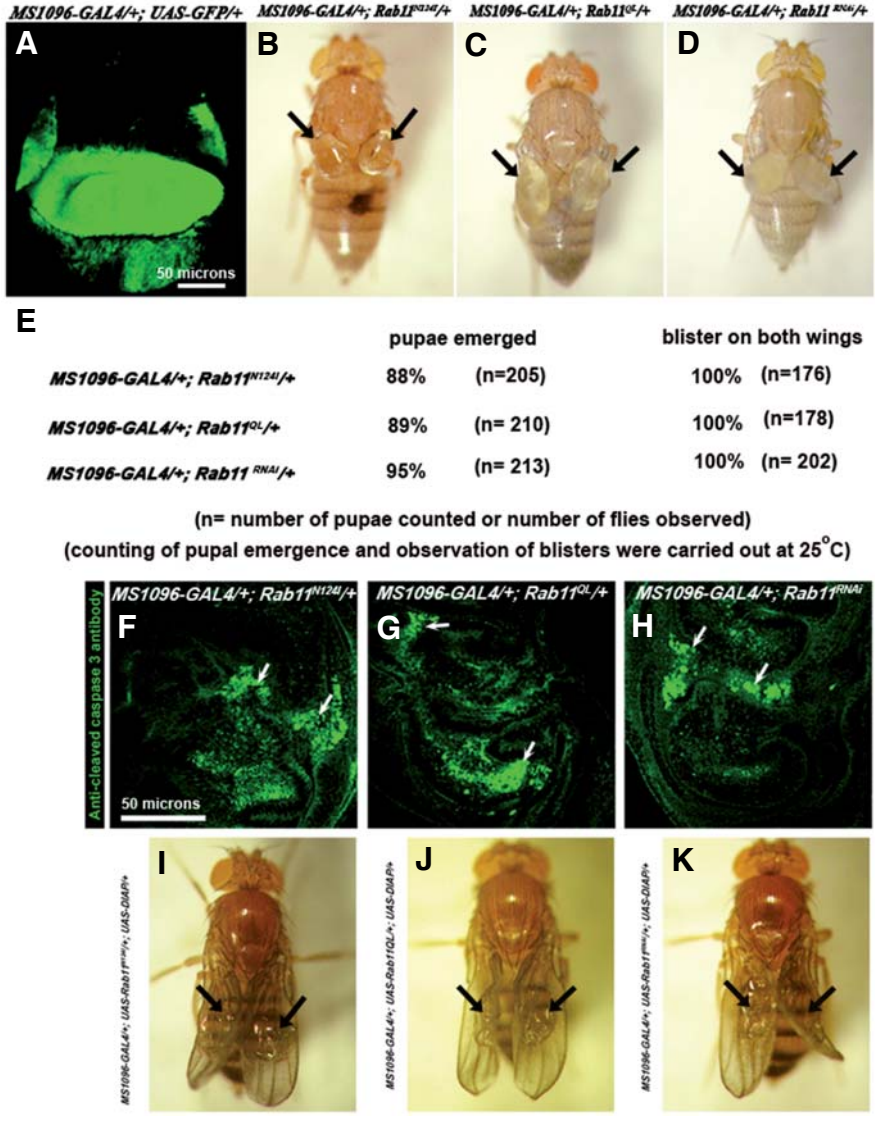


Fig. 5. The expression of dominant-negative or constitutively-active form of Rab11 or *dsRab11*^{RNAi} in wing pouch produces blisters. *The GAL4 expression pattern driven by* MS1096 *was visualized using a GFP reporter (* **(A)**, *green). Over-expression of mutant* Rab11 *or* Rab11^{RNAi} *leads to the formation of wing blisters (arrows) with reduction in wing size (due to cell death). Quantification of emerged pupae and blister formation are represented* **(E)**. *Blisters are indicated by arrows. Cell death was visualized in* Rab11 *mutant backgrounds as stained by anti-cleaved caspase 3 staining.* **(I-K)** *Showing blisters with an increase in wing size when mutant* Rab11 *was co-expressed with an anti-apoptotic gene,* DIAP. *Scale bar, 50 μm.*

rosine antibody (Muller and Wieschaus, 1996) demonstrate increase in the apical surfaces (Supplementary S2) indicating a change in cell shape. These results suggest that Rab11 is required for maintenance of cell shape. Recently, it has been shown that integrins maintains cell shape and loss of integrin changes cell shape from columnar to cuboidal which inhibits in establishing proper contact between dorsal and ventral epithelia resulting blisters in the wings of adult (Dominguez-Jimenez *et al.*, 2007). Further, observation of shape defects in over-expressed mutant Rab11 indicates that this protein might regulate cell adhesion which is consistent with the above finding that βPS integrins are accumulated in the cytoplasm of cells expressing *Rab11*^{N124I} or *Rab11*^{QL} (Fig. 4 B,C) instead of being reached to proper sites of adhesion in wild type cells (Fig. 4A).

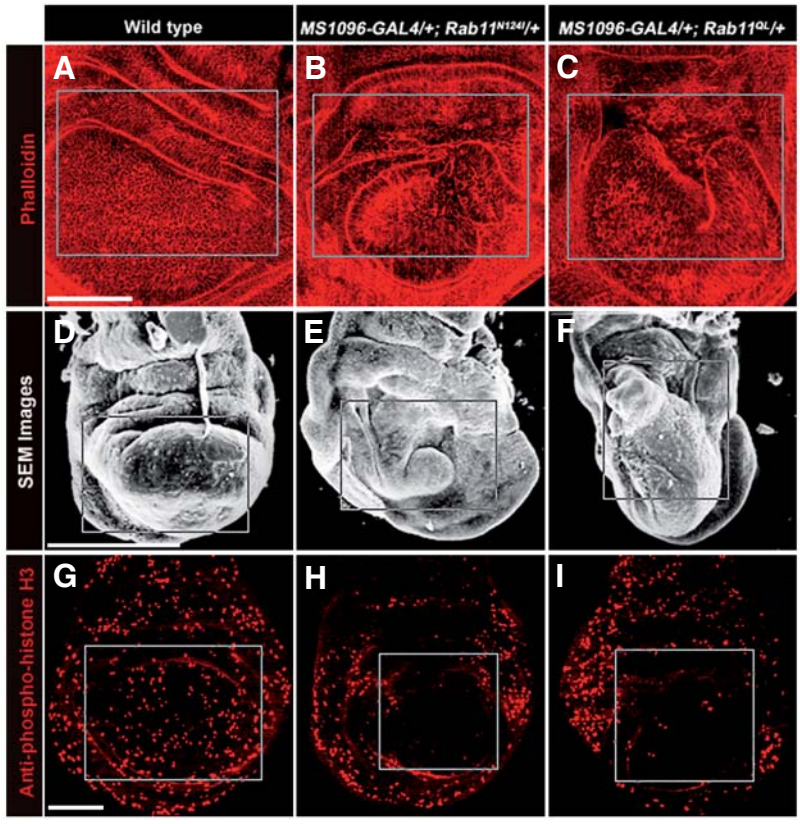


Fig. 6. *Rab11*^{N124I} or *Rab11*^{QL} expression alters wing pouch morphology. *Phalloidin staining shows misfolded wing pouch morphology (boxed area in* **(B,C)***) as compared with wild type (boxed area in* **(A)***). Scanning electron microscopic images showing the misfolded phenotype (boxed area in* **(E,F)***) as compared with wild type (boxed area in* **(D)***). Over-proliferation of cells does not occur in over-expressed mutant* Rab11 *cells as stained with antibody against phosphorylated histone 3 (boxed area in* **(H,I)***, less staining is due to cell death) as compared with wild type (boxed area in* **(G)***). Scale bars:* (A-C) *and* (G-I); *50 μm;* (D-F), *100 μm.*

Over-expression of mutant Rab11 disrupts extracellular matrix assembly and actin-cytoskeleton

Since, the above results suggested abnormal accumulation of βPS integrin in the cytoplasm further we examined whether the matrix integrity has been disrupted in over-expressed mutant Rab11 cells. We used CN6D10 antibody which binds to a special extracellular matrix component secreted between the cells and basal lamina in *Drosophila* imaginal discs (Brower *et al.*, 1987). We found that in over-expressed mutant Rab11 cells, matrix integrity has been changed into a less fibrous appearance (Fig. 7 B,C) as compared to the wild type imaginal disc cells (Fig. 7A). Integrins connect the extracellular matrix to the actin-cytoskeleton through several proteins including α-actinin, talin, tensin and filamin. These proteins act as bridges between actin-cytoskeleton and integrins, among which talin is the most important one (reviewed by Kreis and Vale, 1999; Brakebusch and Fässler, 2003). As in over-expressed mutant Rab11 cells βPS integrins are unable to reach properly in focal adhesion so we further examined the organization of talin and actin-cytoskeleton using anti-talin antibody, phalloidin and an antibody against nonmuscle myosin II, respectively. Dominant-negative or constitutively-activation of Rab11 not only cause a significant alteration of talin but also affects organization of actin-cytoskeleton (Fig. 7 E,F,H,I,K and L) as compared with wild type cells (Fig. 7 D,G and J). Thus, our results strongly suggest that *Rab11* affects matrix assembly and actin-cytoskeleton organization.

Over-expression of mutant Rab11 makes distinct shapes in adherens and septate junctions in wing imaginal disc cells

To investigate the role of Rab11 in cell junctions, we examined the subcellular localization of adherens junctions (AJ) components, DE-cadherin and Arm. The localization of both the proteins were unchanged except that diameter shape of the apical ends of the mutant cells was increased (Fig. 8 B,C,E,F) in comparison to wild type (Fig. 8 A,D) in spite of the fact that Rab11 has been shown to control localization of AJs in epithelium of the dorsal thorax (notum) of pupae (Langevin *et al.*, 2005). Our results were further supported by the observation of Classen *et al.*, 2005 that no gaps in larval wing imaginal discs were formed when *Rab11*^{SN} were expressed. Unlike the vertebrates TJs, which are apical to the AJs, in *Drosophila* they form basal to the AJs on the lateral plasma membrane of the epithelial cells. They are called septate junctions (SJs). SJs are marked by the enrichment of fly proteins like Discs large (Dlg), Coracle (Cora), Scribble (Scrib), Discs lost (Dlt), Lethal giant larvae (Lgl) and Neurexin IV (NeurIV) (reviewed in Knust and Bossinger, 2002). These proteins describe the apical limit of the basolateral membrane domain, along which the transmembrane protein Fas3 is located. The localization of basolateral proteins, Dlg and Fas3 was unaffected but only shapes were changed increasing apical surface area (Fig. 8 H,I,K and L) as compared to the wild type (Fig. 8 G,J). Therefore, our results provide evidence of *Rab11* regulation in maintaining the cell shape in respect to the adherens and septate junctions in wing imaginal disc.

Rab11 genetically interacts with βPS integrin

Since Rab11 has been found in βPS integrin containing recycling endosomes and in post/trans-Golgi vesicles and alterations in Rab11 activity causes abnormal accumulation of βPS integrin in the cytoplasm, it was examined whether Rab11 genetically

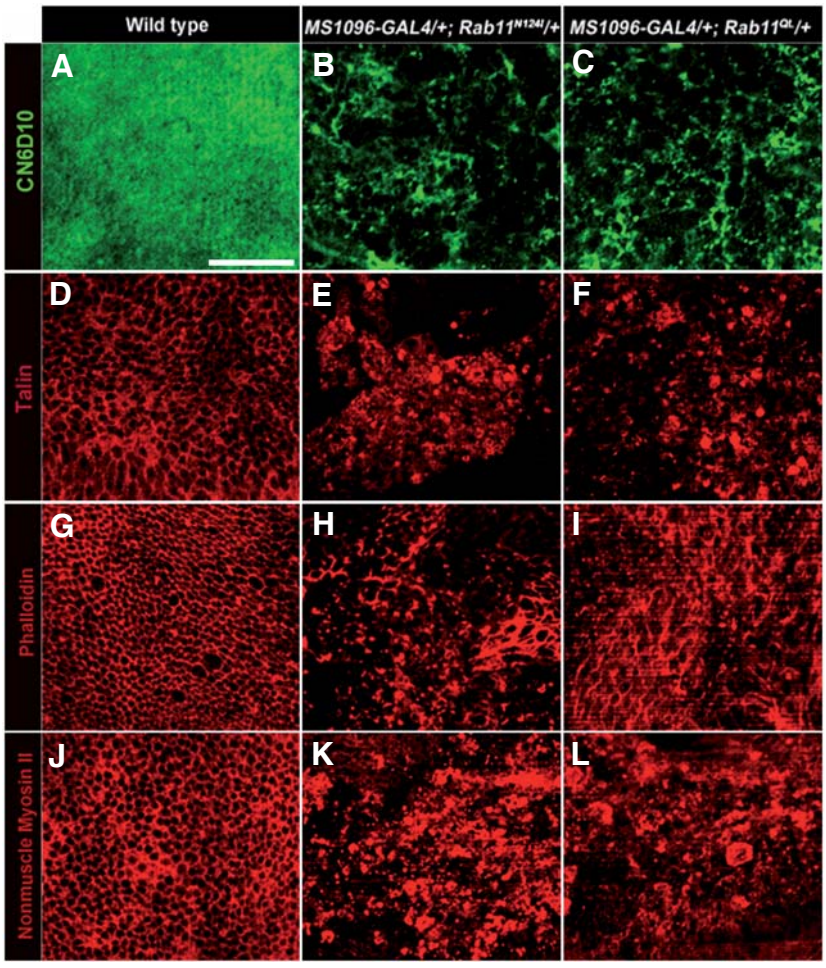


Fig. 7. *Rab11*^{N124I} or *Rab11*^{QL}-expression in wing pouch cells disorganizes matrix integrity, focal adhesion component and actin-cytoskeleton. *Over-expression of dominant-negative or constitutively-active Rab11 disorganize matrix integrity (* **(B,C)** *compared with wild type in* **(A)***) as detected by CN6D10 antibody. In wild type imaginal disc, talin is expressed in a specific manner* **(D)** *and it is disrupted in over-expressed mutant* Rab11 *cells* **(E,F).** *Organization of actin-cytoskeleton was visualized by phalloidin staining, over-expressed mutant* Rab11 *cells show disorganized cytoskeleton* **(H,I)** *as compared with wild type in* **(G)**. *Nonmuscle myosin II staining also reveals its disorganization* **(K,I** *with* **J)** *like phalloidin staining. Scale bar, 10 μm.*

interacts with βPS integrin. Since null mutants of *Rab11* gene (*Rab11*$^{EP(3)3017}$ and *Rab11*$^{l(3)j2D1}$) are lethal and homozygous *Rab11* mutant clones do not survive, we used one hypomorphic allele of *Rab11* (*Rab11*^{mo}) (Alone *et al.*, 2005) which is homozygous viable and do not show blisters in both heterozygous (data not shown) or homozygous condition in wings (Fig. 9 A,B). Gene encoding βPS integrin is *myospheroid* (*mys*) located on X-chromosome and two hypomorphic alleles of *mys*; *mys*^{b43} (Jannuzi *et al.*, 2004) and *mys*^{nj42} (Wilcox *et al.*, 1989) are homozygous viable. Both alleles are hypomorphic and do not show blistering in adult wings in hemizygous (data not shown) or homozygous conditions (Fig. 9 C,D) but shows blister in *Rab11*^{mo} mutant background. Female flies with one copy of *Rab11*^{mo} and double copy of *mys* show blistering in their wings (Fig. 9 F,H) and males occasionally showed blistering when both alleles were in single copy (Fig. 9 E,G) (Quantification of blisters in different genetic backgrounds are represented in Fig. 9M), but double copy of *Rab11*^{mo} in double copy or single copy *mys* backgrounds are lethal, which indicates an impairment of essential *in vivo* interaction between Rab11 and βPS integrin in *Drosophila*. Further, it was confirmed that blister phenotypes is not due to the interaction between *mys* allele and *TM6B* balancer but between *mys* and *Rab11* alleles only as single or double copy of *mys* allele with *TM6B* balancer (*mys/+*; *+/TM6B* or, *mys/mys*; *+/TM6B*) flies do not show any blister in wings (Fig. 9 I-L). This result suggests that *Rab11* is essential for integrin function in *Drosophila* and this is consistent with the findings that Rab11 mediates βPS integrin trafficking.

Discussion

Localization of Rab11 to different membrane compartments in Drosophila *wing disc epithelium indicates its exocytic and transcytic functions*

Various studies using mammalian epithelial cell lines have elucidated the exocytic and recycling pathways for apico-basolateral plasma-membrane proteins and have identified components that guide the proteins along these pathways. These studies have shown that Rab11 localizes to the Golgi apparatus, post-Golgi vesicles and REs, indicating an involvement of this GTPase in both exocytic and endocytic pathways (Ang *et al.*, 2004; Chen *et al.*, 1998; Lock and Stow, 2005; Maxfield and Ullrich *et al.*, 1996; Mcfield *et al.*, 2005; Ren *et al.*, 1998;). In this report, we have investigated the subcellular localization of Rab11 in larval wing imaginal disc cells and found that it localizes to the pericentriolar REs and TGN. It is likely that transmembrane proteins in wing imaginal disc cells of *Drosophila* like other systems after synthesis are being transported from TGN to plasma membrane as well as some of the transmembrane proteins are recycled back to plasma membrane after internalization. This is further supported from the results presented here that one of the transmembrane protein, βPS integrin is being transported via Rab11-positive endosomes from TGN to plasma membrane and are recycled back to plasma membrane after endocytosed and reduction of Rab11 function results in failure of its transport. A dynamic association of Rab11 with TGN and REs were studied during *Drosophila* development and differentiation viz; Rab11 is associated with TGN during male meiosis (Giansanti *et al.*, 2007) and in differentiating photoreceptor cells which does not contain REs where Rab11 transport rhodopsin-containing vesicles from TGN to the apical membranes during rhabdomere morphogenesis (Satoh *et al.*, 2005), while during embryonic cellularization, Rab11 localizes both in TGN and pericentriolar REs and membrane trafficking is mediated by Rab11-positive REs to the advancing furrows (Pelissier *et al.*, 2003). On the other hand, sensory organ precursor (SOP) cells contain only pericentriolar REs with functional significance (Emery *et al.*, 2005). In summary, the association of a pericentriolar RE is cell-specific and tightly regulated. Thus, our results on subcellular localization of Rab11 both in TGN

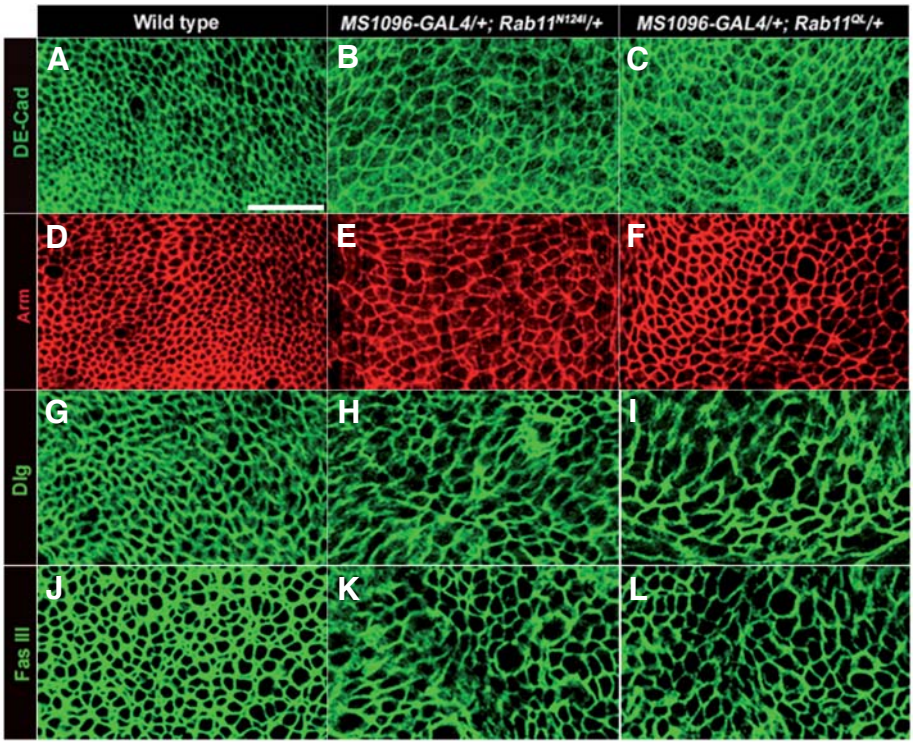
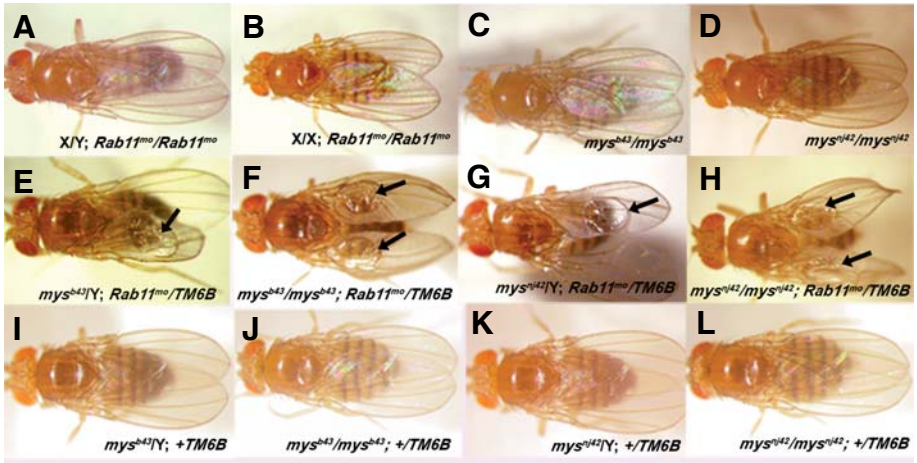


Fig. 8 (Left). Cell outlines marked as adherens and septate junctions are modulated in *Rab11^{N124I}* or *Rab11^{QL}*-expressing tissues. *The localization of adherens junctions markers in over-expressed mutant* Rab11 *cells and in wild type cells remains same as seen by DE-cadherin* **(A-C)** *and β-catenin/Arm* **(D-F)** *staining, however, only apical area has been increased in over-expressed mutant* Rab11 *cells* **(B,C** *and* **E,F)** *as compared with wild type cells* **(A,D)**. *Apical area of septate junctions has also been changed as stained by Dlg and Fas3 antibody* **(H**,**I)** *and* **(K**,**L)** *respectively, as compared with wild type* **(G,J)**. *Scale bar, 10 μm.*



M

	blister on both wings	blister on single wing	normal wings	
mys^{b43}/Y; $Rab11^{mo}/TM6B$	1%	9%	90%	(n=100)
mys^{b43}/mys^{b43}; $Rab11^{mo}/TM6B$	13.9%	30.7%	55.4%	(n=101)
mys^{nj42}/Y; $Rab11^{mo}/TM6B$	1.1%	7.5%	91.4%	(n=90)
mys^{nj42}/mys^{nj42}; $Rab11^{mo}/TM6B$	12%	28%	60%	(n=105)

(observation of blisters and data were taken at 25°C, n= number of flies observed)

Fig. 9 (above). Rab11 genetically interacts with βPS integrin. *Adults of homozygous viable hypomorphic allele of* Rab11^{mo} *male* **(A)** *and female* **(B)** *and two alleles of* mys, mys^{b43} **(C)** *and* mys^{nj42} **(D)** *in homozygous conditions do not show any blisters in their wings. Single copy of* Rab11^{mo} *produces occasional wing blister in a single copy of* ***β****PS integrin mutant background,* mys^{b43} *or* mys^{nj42} *(in males,* **(E**,**G)***) and frequent blisters in double copy of βPS integrin mutant background,* mys^{b43} *or* mys^{nj42} *(in females,* **(F**,**H)***). Blister phenotypes are not because of the interaction between* mys *allele and* TM6B *balancer as single copy of* mys *allele with* TM6B *balancer (*mys^{b43}*/Y;* +/TM6B *or,* mys^{nj42}*/Y;* +/TM6B*)* **(I,K)** *or double copy of* mys *allele with* TM6B *balancer (*mys^{b43}/mys^{b43}; +/TM6B *or,* mys^{nj42}/mys^{nj42}; +/TM6B*)* **(J,L)** *flies do not show any blister in wings. Quantification of blister phenotypes in various genetic backgrounds is presented* **(M)**. *Blisters are indicated by arrows.*

and REs like embryonic cellularization provide evidence of its exocytic trafficking from TGN and transcytic trafficking of βPS integrin through Rab11-positive REs to plasma membrane in wing imaginal disc cells.

Rab11 is essential for cell adhesion, maintenance of cell shape and actin-cytoskeleton organization during Drosophila *wing development*

The integrin family of cell adhesion molecules is conserved among multicellular organisms. The *Drosophila* integrins served as paradigm for genetic studies of cell adhesion proteins during development. Newer proteins involved in executing the function of integrin for its adhesion and signalling pathways are being identified *in vivo* in genetic screens. For about last two decades, it was known that integrin serves two functions in *Drosophila* wings, an early requirement of integrin for signalling events to make the cells efficient for morphogenesis and later during prepupal apposition it executes a structural role where basal surfaces of dorsal and ventral epithelia come in close contact via integrin adhesion (Brabant *et al.*, 1996; Fristrom *et al.*, 1993; Wilcox *et al.*, 1989;). Recently, third function of integrin in maintaining columnar cell shape which is essential for proper contact and adhesion with the cells of opposing wing surfaces during early stages of wing development has been established. Loss of integrin function changes cell shape from columnar to cuboidal which inhibits in establishing proper contact between dorsal and ventral epithelia resulting blisters in the wings of adult fly (Dominguez-Gimenez *et al.*, 2007). In this study, we have indentified and characterized the role of a small GTP-binding protein, Rab11, in executing the known function of integrin through its trafficking during wing development. The data suggest that Rab11 is involved in transcytic and exocytic trafficking of βPS integrin and it is noted that altered Rab11 activity results in failure of βPS integrin transport to its proper adhesion site and accumu-

lated abnormally in the cytoplasm as well as it affects matrix assembly too. The results further reveal that altered Rab11 activity increases the apical area of cells in the wing pouch and changes cell shape (See Supplementary Fig. S2 and Fig. 8). Talin, one of the most important molecule, is used by integrin in making connection of extracellular matrix to the actin-cytoskeleton (reviewed by Brakebusch and Fässler, 2003; Kreis and Vale 1999). Alterations in Rab11 activity also affects proper distribution of not only talin but also actin-cytoskeleton as revealed by phalloidin and non-muscle myosin staining (See Fig. 7). Thus, our results provide evidence that *Rab11* is essential for cell adhesion, maintenance of cell shape and actin-cytoskeleton organization during *Drosophila* wing development.

Rab11 interacts with βPS integrin to regulate cell shape changes underlying epithelial morphogenesis during wing development

Integrins have been shown to interact with different GTPases to regulate morphogenesis, as Rho family of GTPases have been found to interact with integrin during cell spreading and migration in cell lines (reviewed by Schwartz and Shattil, 2000). In epithelial morphogenesis during *Drosophila* wing development Dcdc42 interacts with integrin to form wing blisters (Eaton *et al.*, 1995). Our results for the first time present evidence of the role of another small monomeric GTPase *DRab11* in wing blister formation and this GTPase genetically interacts with βPS integrin underlying epithelial morphogenesis during wing development as it is evident that two hypomorphic alleles ($Rab11^{mo}$ and *mys*) after interaction form blisters in adult wings (See Fig. 9).

The molecular and cellular functions of Rab family members are conserved among eukaryotes (Pereira-Leal and Seabra, 2001). Integrin-mediated adhesion with the extra cellular matrix components is important and plays a role in tumour metastasis. A blistering phenotype is also found in mammals due to lack of integrin-mediated adhesion. In humans, loss of integrins causes separation of epidermis from dermis resulting in a group of closely related diseases known as epidermolysis bullosa where patients suffer from skin blistering, gastrointestinal scarring and some forms of muscular dystrophy (McGrath and Eady, 2001; Pulkkinen and Uitto, 1999). Thus, Rab11 mediated integrin function may be a key regulator of normal cellular development in human epidermis as well as cell to matrix assembly and malfunction of that may results in blistering and metastasis of tumours which is yet to be addressed.

Materials and Methods

Fly stocks and genetics

Following stocks were used: $Rab11^{EP(3)3017}$ (Abdelilah-Seyfried *et al.*, 2000), $UAS\text{-}Rab11^{N124I}$, $UAS\text{-}Rab11^{RNAi}$ and *UAS-CFP-Golgi* (Satoh *et al.*, 2005; gifts from D. Ready), $UAS\text{-}Rab11^{QL}$ (Emery *et al.*, 2005; a gift of M. Gonzalez-Gaitan), $Rab11^{mo}$ (Alone *et al.*, 2005), mys^{b43} (Jannuzi *et al.*, 2004) and $mys^{\eta 42}$ (Wilcox *et al.*, 1989), Other stocks were obtained from the Bloomington Stock Centre.

Generation of Rab11 mutant clones

Somatic clones were generated by using the FLP/FRT system (Xu and Rubin, 1993). To generate *Rab11* mutant clones in wing, larvae of the genotype $yw\,P\{ry^{+}t7.2hsFLP\}22;\,Rab11^{EP(3)3017}P\{ry[+t7.2]=neoFRT\}82B/P\{ry[+t7.2]=neoFRT\}\,82B\,Ubi\text{-}GFP^{nls}$ were given heat shock (37^0C) for 45/60/90 min at 48/72/96 h after egg laying and were subsequently kept at 25ºC until third instar larval stage. The wing discs of third instar larvae (96 h) were dissected in phosphate-buffered saline (PBS), pH 7.4, fixed in 4% paraformaldehyde for 20 min at room temperature (RT) followed by washing three times in PBS for 10 min each and mounted in DABCO and the images were taken on a BioRad MRC Confocal microscope.

Immunostaining

Wing imaginal discs from third-instar larvae were dissected in PBS and fixed in 4% paraformaldehyde/PBS for 20 min and antibody staining was performed by standard procedures. The primary antibodies were used at the following concentrations: rabbit anti-Rab11 (Alone *et al.*, 2005; 1:200), rat anti-DE-cadherin (DCAD2, gift from H. Oda; 1: 100), rabbit anti-Rab5 (gift from G. Gonzales; 1:50), rabbit anti-Rab7 (gift from G. Gonzales; 1:25), mouse anti-βPS integrin (CF6G11, DSHB; 1:10), mouse monoclonal anti-talin (Brown *et al.*, 2002; 1:5), rabbit polyclonal anti-phospho-histone 3 (Ser10) antibody (Upstate; 1:500**)** rabbit anti-nonmuscle myosin II (gift from R. Karess; 1:500), monoclonal CN6D10 that binds to a special component of extracellular matrix (Brower *et al.*, 1987; 1:500), mouse monoclonal anti-phosphotyrosine (Santa Crutz; 1:100), mouse anti-Arm (N27A1, DSHB; 1:50), mouse anti-Fas3 (7G10, DSHB; 1:50), mouse anti-Dlg (4F3, DSHB; 1:10), mouse anti-GFP (Bangalore Genei; 1:100), mouse anti-γ-Tubulin (Clone GTU-88, Sigma; 1:250), rabbit polyclonal cleaved caspase-3 (Sigma; 1:500), mouse anti-p120 Golgi (7H6D7C2, Calbiochem; 1:200), rabbit anti-γ-Tubulin (gift from Y. Zheng; 1:400). The Cy3-coupled secondary antibodies were from Sigma (1:100) and Alexa-488-coupled secondary antibodies were from Molecular Probes (1:200). The samples were mounted in DABCO.

Phalloidin staining

To stain actin filaments, the third instar wing imaginal discs from larvae were dissected in PBS; pH 7.4 followed by fixation in 4% paraformaldehyde/PBS for 20 min at room temperature (RT) and extensive washing for 5X5 min in PBS. Then tissues were permeabilized by washing with PBT (PBS, 0.1% Triton-X100) 3X10 min each followed by washing again in PBS 5X5 min. Finally, tissues were incubated in 1:1000 dilution of PBS/Alexa Fluor-594 phalloidin (Molecular probes) for 30 min and washed in PBS 5X5-6 min each and mounted in DABCO.

Scanning electron microscopy

Wing imaginal discs from third-instar larvae were dissected in PBS at RT and fixed in 2.5% glutaraldehyde in 0.05M cacodilate buffer pH 7.4 for 2 h at 4ºC followed by washing 3X15 min each in 0.1M cacodilate buffer pH 7.4 at 4ºC. The samples were then postfixed in 2% OSO_4 in 0.1M cacodilate buffer pH 7.4 for 1 h at 4ºC followed by serial dehydration in ascending grades of ethanol 3X10 min at 4ºC. Then the samples were air dried followed by mounting on a stud in desired orientations and vacuumed for 15 min. Finally, the samples were coated with gold and analysed on Hitachi S-530 scanning electron microscope at 15 kV.

Image scanning and processing

Immunostained preparations/samples were scanned under a BioRad MRC1024 confocal microscope using Nikon 40X (1.4NA) and‘60X (1.4NA) oil immersion objective. To minimize bleed through, double stained samples were scanned sequentially and then merged. Co-localization study was analyzed using Carl Zeiss LSM 510 Meta Software. Captured images were processed in BioRad MRC1024 confocal software and all images were assembled using Adobe Photoshop. Quantification of co-localization was carried out using image J.

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