Cell polarity and membrane trafficking
CROSS-JOURNAL FOCUS

Cell polarity and membrane trafficking

EDITORS

David del Alamo
Editor
david.delalamo@embo.org | T +49 6221 8891 309
David received his PhD. from the Madrid’s Autónoma University where he studied proximal-distal patterning in Drosophila with Fernando Díaz-Benjumea. As a postdoc, he continued working with Drosophila, first in Marek Mlodzik’s lab (Mount Sinai School of Medicine, New York) on the mechanisms of epithelial planar cell polarity generation, and then with Francois Schweisguth (Institut Pasteur, Paris) where he focused on the modulation of Notch signalling in lateral inhibition. David joined The EMBO Journal in 2011.

Andrea Leibfried
Editor
andrea.leibfried@embo.org | T +49 6221 8891 417
Andrea worked with Jan Lohmann on stem cell maintenance in the plant Arabidopsis before moving to the field of trafficking. In 2009 she obtained her PhD from the Université Pierre et Marie Curie in Paris, for which she studied DE-Cadherin trafficking in Drosophila with Yohanns Bellaiche at the Curie Institute. She then went to Anne Ephrussi’s lab at the EMBL in Heidelberg to work on oocyte polarity and mRNA trafficking in Drosophila. Andrea joined The EMBO Journal in 2013.

Barbara Pauly
Editor
pauly@embo.org | T +49 62218891109
Barbara joined EMBO Reports in September 2008. She completed her PhD at the University of Munich, focusing on signal transduction in the fresh water polyp Hydra. She worked at the University of California at Berkeley as a post-doctoral researcher, studying the role of the actin cytoskeleton in endocytosis in mammalian cells.

Roberto Buccione
Editor
r.buccione@embomolmed.org | T +49 6221 8891 310
Roberto Buccione completed his PhD at the University of L’Aquila, Italy studying the process of oogenesis in mammals. After continuing these studies as a post-doctoral researcher at the Jackson Laboratory, Bar Harbor ME, USA, he joined the Mario Negri Sud research institute in S. Maria Imbaro, Italy, where he lead a research group focused on the cell biology of tumour cell invasion. He joined EMBO Molecular Medicine as a Scientific Editor in October 2012.
**Articles**

**TLR sorting by Rab11 endosomes maintains intestinal epithelial-microbial homeostasis.**
DOI: 10.15252/embj.201487888 | Published 25.07.2014

**In vitro reconstitution of a highly processive recombinant human dynein complex.**
Schlager MA, Hoang HT, Urnavicius L, Bullock SL, Carter AP.
DOI: 10.15252/embj.201488792 | Published 01.07.2014

**EGFR controls IQGAP basolateral membrane localization and mitotic spindle orientation during epithelial morphogenesis.**
Bañón-Rodríguez I, Gálvez-Santisteban M, Vargas-Jaureguí S, Bosch M, Borreguero-Pascual A, Martín-Belmonte F.
DOI: 10.1002/embj.201385946 | Published 13.01.2014

**The kinesin KIF16B mediates apical transcytosis of transferrin receptor in AP-1B-deficient epithelia.**
Pérez Bay AE, Schreiner R, Mazzoni F, Carvajal-González JM, Gravotta D, Perret E, Lehmann Mantaras G, Zhu YS, Rodríguez-Boulán EJ.
DOI: 10.1038/emboj.2013.130 | Published 07.06.2013

**Review**

**A new pathway for mitochondrial quality control: mitochondrial-derived vesicles.**
Sugiura A, McLelland GL, Fon EA, McBride HM.
DOI: 10.15252/embj.201488104 | Published 08.08.2014
TLR sorting by Rab11 endosomes maintains intestinal epithelial-microbial homeostasis

Shiyian Yu1, Yingchao Nie2, Byron Knowles3, Ryotaro Sakamori3, Ewa Stypulkowski3, Chirag Patel4, Soumyashree Das1, Veronique Douard4, Ronaldo P Ferraris4, Edward M Bonder1, James R Goldenring3, Yicktung Tony Ip2 & Nan Gao1,5,*

Abstract

Compartmentalization of Toll-like receptors (TLRs) in intestinal epithelial cells (IECs) regulates distinct immune responses to microbes; however, the specific cellular machinery that controls this mechanism has not been fully identified. Here we provide genetic evidences that the recycling endosomal compartment in enterocytes maintains a homeostatic TLR9 intracellular distribution, supporting mucosal tolerance to normal microbiota. Genetic ablation of a recycling endosome resident small GTPase, Rab11a, a gene adjacent to a Crohn’s disease risk locus, in mouse IECs and in Drosophila midgut caused epithelial cell-intrinsic cytokine production, inflammatory bowel phenotype, and early mortality. Unlike wild-type controls, germ-free Rab11a-deficient mouse intestines failed to tolerate the intraluminal stimulation of microbial agonists. Thus, Rab11a endosome controls intestinal host-microbial homeostasis at least partially via sorting TLRs.

Keywords enterocyte; inflammation; intestinal homeostasis; Rab11a; Toll-like receptor

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Immunology

DOI 10.15252/emby.201487888 | Received 11 January 2014 | Revised 12 June 2014 | Accepted 13 June 2014 | Published online 25 July 2014

The EMBO Journal (2014) 33: 1882-1895

Introduction

A finely tuned immuno-surveillance system that exquisitely balances immuno-responsive and immuno-repressive activities is necessary for microbe–host homeostasis in animal tissues. Genetic and environmental factors that disrupt this balance may underlie various immunological disorders including the inflammatory bowel diseases (IBDs). In mammals, the postnatal intestinal epithelial cells (IECs), after transitioning from a relatively germ-free fetal environment, immediately interact with enteric microbes and participate in immune surveillance against luminal pathogenic stimuli (Artsis, 2008; Maynard et al., 2012). Mature human IECs appear to use specific pathogen pattern recognition receptors such as the Toll-like receptors (TLRs) to balance immune tolerance and immune response, depending on specific cellular localization of the receptors (Abreu, 2010). In cultured human colon epithelial cells, bacterial cytosine-guanine (CpG) stimulation of apically localized TLR9 from the luminal side induced tolerance to subsequent microbial agonist stimulations, whereas basolateral stimulation of TLR9 provoked NF-κB activation and cytokine production (Lee et al., 2006). In contrast, the exclusive basolateral localization of TLR5 in IECs appeared to facilitate this sensor to only respond to the invaded bacterial flagellin protein after barrier function impairment (Gewirtz et al., 2001; Rhee et al., 2005). Furthermore, apical localization of TLR4 was described in human colon epithelial cell lines, and this receptor changed its subcellular localization upon ligand stimulation (Cario et al., 2002). In the small intestine, TLR4 signaled from endosomes in response to its internalized ligand lipopolysaccharide (LPS), allowing differentiation between various types of LPS (Hornef et al., 2003). In addition to receptor compartmentalization, proteolytic cleavage of TLR9 in endolysosomal compartment provides another crucial control for proper receptor activation in immune cells (Ewald et al., 2008; Park et al., 2008). However, the absolute requirement of this proteolytic processing and the role of cleaved N-terminal domain for TLR9 activation are still under intensive studies (Peter et al., 2009; Mouchess et al., 2011; Onji et al., 2013).

Since adverse activation of microbial sensors could elicit unwanted immune responses driving intestinal pathogenesis (Leahart et al., 2007; Fukata et al., 2011), there is a clear imperative to understand the IEC-intrinsic sorting units controlling the proper compartmentalization and activation of TLRs. A recently reported Crohn’s disease risk locus at chromosome 15q22 (dbSNP ID: rs17293632) is adjacent to the human RAB11A (Franke et al., 2010). This gene encodes a small GTPase representing one of the most prominent components of a special endosomal subpopulation—the recycling endosome (Goldenring, 2013). Studies in cultured cell...
lines suggested that the Rab11A endosome engages in intense membrane recycling and sorting, and connect the endo- and exocytic pathways (van Lijndenoor, 2006). In cultured human colonic epithelial cells, Rab11A deletion caused abnormal lumen formation. In mouse intestines, Rab11a expression is increased in IECs during cellular differentiation and maturation (Gao & Kaestner, 2010). Here, we used genetic and biochemical approaches to show that Rab11a endosomal compartment maintains homeostatic TLR9 compartmentalization at steady-state conditions. By doing so, Rab11a appeared to prevent unwanted pro-inflammatory stimuli. Genetic and cell type-specific inactivation of Rab11a in mouse and Drosophila IECs midgut caused aberrant NF-κB activation, inflammatory cytokine production, and IBP phenotypes. Removal of microbial ligands (germ-free) alleviated these phenotypes in the mutants. Unlike wild-type controls, germ-free Rab11aStable mice failed to tolerate intraluminal perfusion of microbial TLR agonists. Our data suggested that Rab11a controls intestinal microbial tolerance at least partially via sorting TLRs.

Results

Rab11a ablation in intestinal epithelia causes inflammation

To study the contribution of Rab11a recycling endosome to intestinal host-microbial homeostasis, we derived a Rab11a floxed (fl) conditional mouse allele. Rab11a global knockout mice (Rab11a+/−) died in utero around the implantation stage (data not shown); therefore, we established IEC-specific Rab11a knockout mice (Rab11afl/fl; Villin-Cre+, or Rab11aStable) using the Villin-Cre transgene. Rab11aStable mice were born at the expected Mendelian ratio; however, at a young age, they exhibited significant running (Fig 1A and B, see asterisk), and an approximately 40% mortality rate at the age of weaning. Male mutant mice showed higher mortality rate than their female counterparts; survivors also displayed higher mortality with aging (data not shown). Both male and female mutant mice had a dilated intestinal lumen and a shortened colon compared with their wild-type littermates (Fig 1C, see asterisks). Western blot confirmed the removal of Rab11a protein from Rab11aStable mouse intestinal epithelia (Fig 1D). In contrast to control intestinal epithelia, where Rab11a was detected by immunohistochemistry in the subapical cytoplasm of villus epithelial cells (top panels, Fig 1E), Rab11a staining was absent from the Rab11aStable intestinal villus epithelia (bottom panels, Fig 1E). Histopathologically, Rab11aStable intestines showed blunted villi, reduced goblet cell numbers, and macrophage infiltration into the submucosa (Fig 1F). In neonatal Rab11aStable mice, the intestinal villi were frequently observed to fuse and branch (see also Supplementary Fig S1A). Pulse-chase BrdU labeling or immunostaining for phosphorylated Histone H3 (pH3) detected increased numbers of cycling crypt cells in Rab11aStable mice at all postnatal (P) stages (Fig 1G). This crypt hyperplasia was consistent with the generally enlarged crypt morphology in these mutants (Fig 1G).

Increased crypt cell proliferation was also detected when Rab11a deletion was induced in adult Rab11afl/fl; Villin-CreERT2 mice by tamoxifen administration (Supplementary Fig S2), suggesting that the hyperproliferative response of crypt cells might not simply be a result of defective intestinal development. Indeed, the differentiation of enterocytes and three intestinal secretory cell lineages was unaffected in Rab11aStable mice (enterocytes and goblet cells shown in Fig 1F, paneth and enteroendocrine cells in Supplementary Fig S3A). Rab11a-deficient enterocytes were still capable to elaborate apical brush borders (Supplementary Fig S3B). Therefore, Rab11a deletion did not appear to disrupt the differentiation of major intestinal cell lineages. However, the epithelial hyperplasia, dysplasia, and immune cell infiltration continued to be present in older survivor Rab11aStable mice (5 months old shown in Supplementary Fig S1B), which showed high histopathology scores indicative of intestinal inflammation (Fig 1H) (Adolph et al., 2013). Finally, tamoxifen induced deletion of Rab11a specifically in Lgr5+ intestinal epithelial stem cells (IESCs, or crypt-based columnar cells, CBCs) in Rab11afl/fl; Lgr5EGFP-IRES-CreER mice increased the proliferation of both the Lgr5+ and the transit-amplifying cells (Fig 1I). No immediate cell death was triggered by inducible Rab11a deletions (data not shown).

To identify the mechanisms underlying the above pathological abnormalities, we performed microarray analysis on neonatal (postnatal day 3, P3) mouse intestines using four independent pairs of RNA samples from Rab11aStable and wild-type littermates (Fig 2A). Inflammatory genes encoding cytokines, chemokines, defensins, and anti-microbial peptides were significantly upregulated as the most enriched gene category in Rab11aStable intestines (Fig 2B and C). Overlapping analyses revealed that 79 and 138 upregulated transcripts in Rab11aStable were shared by two independent mouse enteritis models: transgenic CD98Fl/+ and trinitrobenzene sulfonate (TNBS)-induced enteritis, respectively (Nguyen et al., 2011; Avula et al., 2012) (Fig 2D). These findings from microarray analysis were confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) analyses, with IL6, CXCL1 (human IL8 homolog), and CXCL5 being the most robustly activated genes (Fig 2E). A number of genes downstream of canonical Wnt pathway were not changed. Activation of IL6 signaling pathway in Rab11aStable intestines was further supported by elevated levels of phosphorylated Stat3 (pStat3), a downstream effector of IL6 (Fig 2F). Luminescent multiplex cytokine/chemokine assays identified elevated levels of IL-6, IL-1β, and MCP1 in Rab11aStable mouse serum (Fig 2G), supporting an activated systemic inflammatory response in these mice.

Professional immune cells and IECs are both capable of producing inflammatory cytokines including IL-6 (Kusugami et al., 1995; Vinderola et al., 2005). To identify the sources of these cytokines, we first performed IL-6 immunofluorescent analyses. Higher IL-6 immunoreactivities were detected in Rab11aStable intestinal epithelia compared with the control epithelia (Fig 2H). The strongest IL-6 signal in wild-type epithelia was found in immune cells resident in the lamina propria (arrowheads in Fig 2H). To determine whether Rab11a-deficient IECs were the origins of observed cytokine production, we performed intestinal organoid cultures and directly measured the secreted inflammatory cytokines within the supernatant of culture medium containing live organoids (Fig 2I). After 1 week in culture, Rab11aStable organoids, presumably devoid of lymphocytes, produced higher amounts of IL-6, SDF-1α/CXCL12, and IL-1β measured by ELISA (Fig 2J). Of note, even in culture, Rab11aStable organoids initiated bud outgrowths more rapidly and contained a larger population of proliferative cells than wild-type organoids (Fig 2I). Therefore, the isolated Rab11aStable crypts might have contained more progenitor cells to start with. Deletion of Rab11a in cultured Rab11afl/fl;Villin-CreERT2 organoids through
Rab11 controls intestinal homeostasis
Shiyen Yu et al

Figure 1. Rab11a ablation in mouse IECs caused enteritis.
A–B Rab11a<sup>−/−</sup> mice showed running and postnatal growth retardation (see asterisks).
C Rab11a<sup>−/−</sup> mice (red asterisks) had dilated small intestines and shortened colons.
D Western blots confirmed Rab11a ablation.
E Immunohistochemistry confirmed Rab11a deletion in Rab11a<sup>−/−</sup> mouse IECs. In wild-type controls (top panels), Rab11a was detected in subapical cytoplasmic region. Scale bars, 10 μm.
F Early postnatal Rab11a<sup>−/−</sup> intestines contained blunted villi, reduced goblet cells, and macrophage infiltrations. Enterocytes were detected by alkaline phosphatase staining. Scale bars, 10 μm.
G BrdU labeling and pHH3 staining identified significantly increased crypt epithelial cell proliferation in Rab11a<sup>−/−</sup> intestines at all postnatal stages examined. Note that Rab11a<sup>−/−</sup> intestinal crypts were enlarged in representative 4-week-old and P6 mice. Scale bars, 10 μm. ***P < 0.001.
H Rab11a<sup>−/−</sup> intestines showed higher histopathology scores indicative of intestinal inflammation, compared with wild-type littermates before and after weaning. ***P < 0.001.
I Inducible Rab11a deletion in Lgr5<sup>+</sup> cells caused increased proliferation in both IESCs and transit-amplifying cells. Five to 10 mice of each genotype were used in individual phenotypic analysis. Scale bars, 20 μm. ***P < 0.001.

administering tamoxifen elicited a less potent increase of IL-6 secretion (Fig 2K), suggesting that loss of Rab11a triggered an epithelial cell-autonomous cytokine production.

**Rab11 depletion in Drosophila enterocytes caused IBD-like phenotype**

The mouse Villin promoter-driven Rab11a deletion described above targeted all IEC cell types. It was not possible to distinguish the cell-autonomous mechanism from non-autonomous mechanism that was responsible for triggering the inflammatory responses. We employed an intestinal cell type-specific knockdown approach in *Drosophila* midgut—a tissue equivalent to mammalian intestine—using RNAi against Rab11, the only Rab11a homolog in fly genome. This system enabled us to inducibly deplete Rab11 in a particular IEC cell type via tightly controlled Gal4 drivers: the Delta promoter-Gal4 (DIGal4) for IESCs, the Su(H) binding site-Gal4 [Su(H)Gal4] for enteroblasts (EBs), the escargot promoter-Gal4 (esgGal4) for IESCs and EBs, and the Myo51A promoter-Gal4 (Myo51AGal4) specific for enterocytes (Michelli & Perrimon, 2006; Zeng et al, 2010; Jiang et al, 2011) (Fig 3A). Entire midguts from cell-specific Rab11 RNAi and control RNAi adult flies were analyzed for pHH3<sup>+</sup> mitotic cells. Remarkably, enterocyte-specific Rab11 knockdown, mediated by the Myo51AGal4 driver, caused the highest, nearly 100-fold, increase of mitosis in IESCs, whereas the esg and Su(H) drivers caused only modest increases of IESCs proliferation.
(Fig 3B). In addition to the Rab11TRiP RNAi line, two additional Rab11 RNAi lines (Rab11v108382 and Rab11v1208362) targeting distinct Rab11 sequences led to similar phenotypes after crossing with the Myo1AGal4 driver (Fig 3C), suggesting the phenotype was Rab11-specific. Epithelial organization was determined by confocal microscopy based on Myo1A-driven GFP expressed only in enterocytes and the β-catenin (Armadillo) staining that highlighted the cell membrane (Fig 3D–I). In wild-type midgut, the IESC-EB cell normally contained 1–2 cells (Fig 3D, arrows). However, in Myo1AGal4-driven Rab11 RNAi midguts, the nests were composed of increased number of cells (Fig 3F and H), with the Rab11v1208362 line showing the strongest phenotype with the highly expanded cell nests occupying most of the gut epithelium (Fig 3H). These cell nests were expanded from Rab11 wild-type IESC as they did not contain Myo1AGal4-driven GFP. Visualized from sagittal sections, these expanded cell nests continued to occupy the basal side of the

---

**Figure 2. Rab11a-deficient IECs activated and overproduced inflammatory cytokines.**

A. Microarray analyses using four pairs of neonatal (P0) Rab11a−/− and wild-type littermate control intestinal samples. Upregulated inflammation-related genes clusters are shown in red, including Dfel39, Il1b, Lysl, Ifnγ, and Il1f4, with corresponding fold changes (FC) shown relative to controls.

B. Functional annotation clustering of the most significantly upregulated categories in Rab11a−/− samples.

C. Representative cytokines, chemokines, and anti-microbial peptides were shown to be upregulated in Rab11a−/− samples.

D. The mouse IECs. In wild-type controls (top panels), Rab11a was detected in subapical cytoplasmic region. Scale bars, 100 μm.

E. qRT-PCR analyses confirmed cytokine gene activations. Note that several Wnt pathway target genes, Lgr5, Atoh1, and Olfm4, were not changed. *P < 0.05, **P < 0.01.

F. Increased pStat3 levels in Rab11a−/− intestines. Graph represented normalized pStat3 levels against total Stat3 levels. **P < 0.01 for three independent mice of each genotype.

G. Multiplex cytokine assays showed that Rab11a−/− mice had higher serum levels of inflammatory cytokines.

H. IL-6 immunofluorescent analyses identified its overexpression by Rab11a-deficient IECs. Arrowheads point to immune cells strongly expressing IL-6 in both control and mutant tissues. Lu, lumen. Scale bars, 15 μm.

I. The proliferative cell numbers in cultured Rab11a−/− organoids increased almost twofold, as determined by phH3 and EdU double labeling. Scale bars, 20 μm.

J. Multi-analyte cytokine ELISA detected higher levels of IL-6, IL-1β, and SDF-1α in the supernatants of Rab11a−/− organoid cultures (see arrowheads). *P < 0.05, **P < 0.01.

K. Inducible Rab11a deletion by tamoxifen in cultured Rab11a+/+; V inflict-ER organoids induced the average IL-6 production by twofold.
epithelium (Fig 3E, G and I), illustrating an overall benign epithelial organization except for an increased precursor cell number due to IESC hyperproliferation. These results suggested that Rab11 depletion from enterocytes triggered neighboring IESC division, possibly through non-autonomous mechanisms.

Using qRT-PCR, we analyzed a variety of ligands and growth factors, including activators of the Wnt, JAK-STAT, and EGFR signaling pathways (Jiang & Edgar, 2012) known to be critical for IESC proliferation in adult Drosophila midgut. Among these factors examined, Upd3, a Drosophila IL-6 equivalent ligand that activates the JAK-STAT pathway (Zhou et al, 2013), showed the highest increase (~600-fold) in its expression comparing to controls (Fig 3J). We used an Upd3 promoter-driven lacZ reporter line (Zhou et al, 2013) to determine the identity of cells within the midgut that contributed to this high level of Upd3 expression. LacZ expression was markedly increased in Rab11 RNAi midguts, with a pattern identical to enterocytes that were marked by Myo1A-driven GFP (Fig 3K). Collectively, the above results from mouse intestines and Drosophila midgut strongly suggested that Rab11-deficient enterocytes triggered cell-autonomous cytokine productions that indirectly impacted IESC proliferation.

**Activation of NF-κB and MAPK pathways in Rab11a^{AGal} intestines**

To identify the inflammatory signaling pathways that were upstream of the epithelial cytokine responses in Rab11a-deficient intestines, we analyzed NF-κB, MAPK, and JNK pathways, known to be critically involved in inflammatory and stress responses (Pasparakis, 2009; Arthur & Ley, 2013). Western blots detected significantly elevated P65 and RelB protein levels in Rab11a^{AGal} mice at all postnatal stages, but not at fetal stages (Fig 4A). Inducible deletion of Rab11a in adult Rab11a^{AGal}; Vil-CreER mice by tamoxifen injection also increased the levels of P65 and RelB (Fig 4B). These changes in NF-κB protein levels likely reflected a specific response to the loss of Rab11a, as deletion of Rab8a, another small GTPase regulating apical membrane trafficking in IECs (Sato et al, 2007), did not increase P65 or RelB levels (Fig 4C). NF-κB activation in Rab11a^{AGal} mouse intestines was also supported by elevated nuclear P65 levels from fractionated tissue lysates (Fig 4D). Abnormal activation of NF-κB was reported to cause intestinal inflammation (Zhang et al, 2006; Vereecke et al, 2010; Vlantis et al, 2011), similar to the phenotype of Rab11a^{AGal} mice. Interestingly, we found that, during intestinal development in wild-type mice, P65 and RelB protein levels attenuated from late gestation stage. Rab11a levels increased during the time course, illustrating a reverse correlation (Fig 4E). Immunohistochemistry for P65 showed that postnatal Rab11a^{AGal} intestinal villus epithelia, unlike wild-type littermates, continued to strongly express this master activator of inflammation (Ghosh & Hayden, 2008) (Fig 4F). In addition to NF-κB, activation of MAPK (Fig 4G) but not JNK pathway (data not shown) was detected in Rab11a^{AGal} intestines, suggesting that the molecular alterations in mutant tissues did not simply reflect a generalized cell stress.

To determine whether the NF-κB pathway was required for the increased cytokine production and crypt cell proliferation, we treated Rab11a^{AGal} organoids with an NF-κB pathway inhibitor, BAY11-7082, which irreversibly inhibits the phosphorylation of IκBα. BAY11-7082 caused significant reductions in IL-6 production and cell proliferation in Rab11a^{AGal} organoids (Fig 4H and I). These inhibitory effects were as strong as those elicited with an IL-6 neutralizing antibody (Fig 4I). Although IL-6 recombinant proteins stimulated the proliferation of wild-type organoids, the resulting proliferative rate was still lower than Rab11a^{AGal} organoids (Fig 4I), suggesting that additional cytokines might have also contributed to Rab11a^{AGal} hyperplasia.

**Loss of Rab11a impacted the homeostatic TLR9 intracellular distribution**

As microbial sensors in IECs, TLR signaling constitutes the primary link between enteric microbes and epithelial cell-intrinsic NF-κB signaling. The elevated levels of phosphorylated Erk (Fig 4D), another mediator of TLR9 receptor signaling (Lee et al, 2006), and a number of downstream genes of TLR9 signaling, such as defensins and CXCL1 (Lee et al, 2006) (Fig 2C and E), collectively suggested that TLR9 signaling pathway might be activated in Rab11a-deficient IECs. We then explored the potential contribution of abnormal TLR signaling to the observed phenotypes. Despite a normal TLR9 mRNA level detected by microarray and quantitative RT-PCR (Supplementary Fig S4), immunolocalization of TLR9 with a documented antibody (Lee et al, 2004, 2006; Tabela et al, 2006; Palladino et al, 2007) revealed aberrant subcellular receptor aggregations that appeared to be basally (bl) shifted in Rab11a-deficient IECs (bottom panels in Fig 5A and B, and Supplementary Fig S5). Wild-type IECs appeared to be basally (bl) shifted in Rab11a-deficient IECs (lower panel in Fig 5C), suggesting a changed receptor proteolytic processing.

In Rab11a^{AGal} midguts, with a pattern identical to enterocytes that were marked by Myo1A-driven GFP (Fig 5B), or with Rab7+ vesicles (Supplementary Fig S5). Close association of TLR9 aggregates with Lamp2+ (Fig 5B), or with Rab7+ vesicles (Supplementary Fig S5). Wild-type IECs contained primarily small apical (ap) TLR9 puncta in addition to weak cell surface signals (Lee et al, 2006) (top panels in Fig 5A and B, and Supplementary Fig S5). Close association of TLR9 aggregates with Lamp2+ (Fig 5B), or with Rab7+ vesicles (Supplementary Fig S5), was significantly increased in Rab11a-deficient enterocytes, suggesting that an increased amount of TLR9 was retained by endolysosomes.

Western blots for TLR9 detected altered receptor fragmentation patterns in adult Rab11a^{AGal} intestines (male and female, right panel in Fig 5C), suggesting a changed receptor proteolytic processing. The detected multiple TLR9 fragments in wild-type intestines might reflect stepwise receptor processing by cathepsin and asparagines endopeptidase (Ewald et al, 2011). Using a stable TLR9 knockdown (KD) human colonic epithelial Caco2 cell line, we validated the specificity of these endogenous TLR9 fragments (Supplementary Fig S6). This different TLR9 fragmentation pattern was absent in fetal Rab11a^{AGal} intestines (E15.5, left panel in Fig 5C), but emerged from neonatal stages (P6: left panel in Fig 5C; 2-weeks: Fig 5D and Supplementary Fig S6). These results suggested that in the absence of Rab11a vesicles (left panel, Fig 5D); in Rab11a’s absence, TLR9 distribution shifted toward fractions containing late endosome (Rab7) and lysosome (Rab9) (right panel, Fig 5D). Using co-immunoprecipitation (co-IP), we detected increased TLR9 retention by Rab7 endosomes and an elevated TLR9-MyD88 interaction in Rab11a^{AGal} mouse intestines (see arrows, Fig 5E), supporting that in the absence of Rab11a vesicles, increased amount of TLR9 was contained by late endosome and/or lysosome, where TLR9 was reported to be processed and activated in innate immune cells (Ewald et al, 2008; Park et al,
Western blots detected intestines, we analyzed NF upregulation upstream of the epithelial cytokine responses in Rab11a-deficient that postnatal reverse correlation (Fig 4E). Immunohistochemistry for P65 showed stage. Rab11a levels increased during the time course, illustrating a reverse correlation with NF activation. Abnormal activation of NF \(\text{NF}_{\text{B}}\) was marked by Myo1A-driven GFP, similar to the phenotype of Rab11-deficient enterocytes. MAPK and JNK pathways, known for their role in inflammation and cell proliferation, were examined. Rab11afl/fl; Vil-CreER mice by Park et al. (2007) revealed aberrant subcellular receptor aggregations that contributed to this high level of Upd3 expression. LacZ expression was markedly increased in Rab11 RNAi midguts, with a pattern identical to enterocytes that were marked by Myo1A-driven GFP.

The EMBO Journal

Figure 3. Inducible Rab11 depletion in Drosophila enterocytes caused stem cell hyperplasia and cytokine activation.

A. Cell type-specific Rab11 knockdown in Drosophila midguts was achieved using promoter-specific Gal4 drivers shown in red. IESC: intestinal epithelial stem cell; EB: enteroblast; EE: enteroendocrine cell; EC: enterocyte.

B. Entire midguts of control RNAi and Rab11 RNAi flies were counted for pH3\(^*\) mitotic cells. Myo1AGal4-driven enterocyte-specific Rab11 knockdown induced the greatest IESC division. **P < 0.001.

C. Similar phenotypes were detected in three independent Rab11 RNAi lines: v22198, v108382, and Trip, all driven by the Myo1AGal4. Representative pH3\(^*\) staining is shown for v108382 Rab11 RNAi fly midgut. Arrows point to mitotic cells (red). Nuclei were stained by DAPI in blue.

D-I. Confocal images of midguts from control and Rab11 RNAi flies. D, F, H are surface views; and E, G, I are sagittal views. Green cells were Myo1AGal4-driven GFP that marked enterocytes. Membrane was stained by β-catenin (Armadillo) antibody (red), nuclear red was Prospero for enteroendocrine cells, and nuclei were stained by DAPI in blue. The arrows point to IESC-EB cell nests encircled by dotted lines. Scale bars, 20 μm.

J. Increased Upd3 mRNA level was detected by qRT-PCR in Myo1AGal4-driven Rab11-depleted Drosophila midguts.

K. Confocal images of midguts from Upd3-LacZ reporter showed undetectable Upd3 promoter activity in control, but higher reporter activities (red) in Rab11-depleted enterocytes, which were marked by Myo1AGal4-driven GFP (green), suggesting that Rab11 depletion caused cell-autonomous Upd3 activation in enterocytes. Scale bars, 20 μm.
Additionally, when we examined adult germ-free Rab11a<sup> ΔIEC </sup> mouse intestines where microbial TLR agonists were absent, we observed an identical pattern to the one seen in fetal intestines (Fig 5C and F). These data suggested that Rab11a critically contributed to TLR9 sorting in IECs of conventionally housed, that is, the specific pathogen free (SPF), mice.

Notably, in Caco2 cells that feature mature enterocytes (Peterson & Mooseker, 1992), TLR9 was detected by co-IP to be contained in IEC-intrinsic activation of NF-kB and MAP kinase pathways in Rab11a<sup> ΔIEC </sup> mice.
A P65 and RelB levels were elevated in postnatal Rab11a<sup> ΔIEC </sup> intestines, B Inducible Rab11a ablation in Rab11a<sup> ΔIEC </sup>;Vil-CreER mice also elevated P65 and RelB levels, C P65 and RelB levels were not elevated in Rab11a knockout intestines, D Nuclear fractionation assays determined that there was a higher level of nuclear P65 in Rab11a<sup> ΔIEC </sup> epithelia (15.7%) than control epithelia (9.1%), E During the development of wild-type mouse intestines, NF-κB and Rab11a levels showed reverse correlations, F P65 level was drastically decreased in postnatal villus epithelia, but was continuously activated in postnatal Rab11a<sup> ΔIEC </sup> epithelia. Scale bars, 20 μm, G Levels of phosphorylated Erk were increased in Rab11a<sup> ΔIEC </sup>;Vil-CreER mice also elevated P<sub>E</sub>G<sub>12</sub>, and RelB levels were not elevated in Rab11a knockout intestines, H NF-κB inhibitor BAY11-7082 suppressed IL-6 production from Rab11a<sup> ΔIEC </sup> organoids, *P < 0.05, I BAY11-7082 and IL-6 neutralizing antibody suppressed the proliferation of Rab11a<sup> ΔIEC </sup> organoids. Scale bars, 15 μm. *P < 0.05, ***P < 0.001.

Rab11 controls epithelial tolerance to TLR agonists

Abnormal TLR9 activation provides potential mechanism for NF-κB activation and inflammatory response observed in Rab11a<sup> ΔIEC </sup> mice, as compartmentalization of TLR9 in IECs was reported to balance the mucosal tolerance and immune response to microbial stimulations (Wells et al, 2011). It was possible that Rab11a<sup> ΔIEC </sup> intestinal epithelia were leaky causing bacterial activation of basolateral TLRs such as TLR5 (Rhee et al, 2005), but barrier function analyses using radioactive <sup>3</sup>H-L-glucose and <sup>14</sup>C-insulin failed to detect leakage in
RAB11A’s depletion, a sevenfold increase of TLR9 retention by membrane protein isolation assays using stable RAB11A-KD cells mouse splenic dendritic cells (Onji reported in both human colon epithelial cells (Lee & Mooseker, 1992), TLR9 was detected by co-IP to be contained in specific pathogen free (SPF), mice. 

uted to TLR9 sorting in IECs of conventionally housed, that is, the (Fig 5C and F). These data suggested that Rab11a critically contrib-

observed an identical pattern to the one seen in fetal intestines (2008). Additionally, when we examined adult germ-free intestines (IEC) with activated Rab11a+ vesicles was observed in these cells (Supplementary Fig 5D). However, Rab11a and Rab7 were decreased in postnatal villus epithelia, but were continuously activated in postnatal gut 

D Subcellular fractionations showed shifted TLR9 compartmentalization in Rab11a+/IEC toward low sucrose density fractions containing endolysosome. Lane 1–12: 0–50% sucrose fractions (see Materials and Methods). TL: total lysates. XY scatter plots illustrating percentage distribution in each sucrose fraction were generated in Excel on the basis of densitometry measurements by NIH ImageJ.

E Co-IP analyses using mouse intestinal lysates showed increased TLR9 proteins in Rab7+ vesicles and elevated TLR9-MyD88 association (see arrowheads) in Rab11a+/IEC compared to control mice. Asterisk indicates small TLR9 fragments present in Rab7a complexes.

F TLR9 fragmentation pattern was similar between control and mutant adult mice at germ-free condition. Note that the patterns in germ-free intestines were similar to those in E15.5 intestines in (C).

G LysoTracker showed that Rab11a-KD Caco2 cysts contained larger acidic endolysosomal compartments. Rainbow color scale identifies acidic compartments as yellow. Scale bars, 15 μm.

H Rab11a-KD Caco2 cells showed increased IL6 and cIAP2 mRNA levels. ***P < 0.001.

Figure 5. Rab11a deficiency impacted TLR9 distribution, fragmentation, and activation.

A Immunofluorescent staining for TLR9 (green) detected intraepithelial cell aggregations that shifted basally (bl) in Rab11a-deficient IECs. In wild-type IECs, TLR9 was detected as apical (ap) puncta, in addition to cell surface signals. lu: lumen. Scale bars, 10 μm.

B Costaining for TLR9 (green) and Lamp2 (red) showed increased TLR9 aggregations in Lamp2+ compartment. Arrows indicate closely associated two signals. Scale bars, 10 μm. ***P < 0.001.

C Western blots for TLR9 showed changed receptor fragmentation pattern in both male and female adult Rab11a+/IEC mice at SPF conditions. Results represent data from six mice. Note that at E15.5 fetal stage, TLR9 fragmentation pattern was similar between control and mutant intestines. Arrowhead points to an approximately 95 kDa processed TLR9. Lower molecular weight bands (< 40 kDa) may reflect non-specific proteins.

D Subcellular fractions showed shifted TLR9 compartmentalization in Rab11a+/IEC toward low sucrose density fractions containing endolysosome. Lane 1–12: 0–50% sucrose fractions (see Materials and Methods). TL: total lysates. XY scatter plots illustrating percentage distribution in each sucrose fraction were generated in Excel on the basis of densitometry measurements by NIH ImageJ.

E Co-IP analyses using mouse intestinal lysates showed increased TLR9 proteins in Rab7+ vesicles and elevated TLR9-MyD88 association (see arrowheads) in Rab11a+/IEC compared to control mice. Asterisk indicates small TLR9 fragments present in Rab7a complexes.

F TLR9 fragmentation pattern was similar between control and mutant adult mice at germ-free condition. Note that the patterns in germ-free intestines were similar to those in E15.5 intestines in (C).

G LysoTracker showed that Rab11a-KD Caco2 cysts contained larger acidic endolysosomal compartments. Rainbow color scale identifies acidic compartments as yellow. Scale bars, 15 μm.

H Rab11a-KD Caco2 cells showed increased IL6 and cIAP2 mRNA levels. ***P < 0.001.
\textit{Rab11a\textsuperscript{ATEC}}-epithelia, which showed rather stronger barrier function than wild-type littermates (Fig 6A), consistent with formation of apical IEC junctions in the mutants (arrows in Supplementary Fig S3B). However, germ-free \textit{Rab11a\textsuperscript{ATEC}} mice did demonstrate an overall reduction of crypt cell proliferation (Fig 6B and C) and serum IL-6 levels (Fig 6D) compared with \textit{Rab11a\textsuperscript{ATEC}} mice at SPF conditions. Remarkably, germ-free mutants showed reduced mortality and some became capable of breeding (data not shown). Organo- 
doids derived from germ-free \textit{Rab11a\textsuperscript{ATEC}} mice also showed reduced \textit{Il6} expression (Fig 6E), secreted less IL-6 proteins (Fig 6F), and proliferated less (Fig 6G). These data supported the notion that the microscopic status influenced the progression of inflammatory phenotype in \textit{Rab11a\textsuperscript{ATEC}} mouse intestines.

To determine the specific responses of germ-free \textit{Rab11a\textsuperscript{ATEC}} intestinal epithelia to distinct TLR agonists, we performed luminal perfusion analyses in live adult mice using specific ligands for TLR9 (endotoxin-free \textit{Escherichia coli} DNA) and TLR4 (LPS) (Fig 6H). Both wild-type and \textit{Rab11a\textsuperscript{ATEC}} germ-free intestines responded to 4 h of \textit{E. coli} DNA perfusion at molecular level, showing increased levels of phosphorylated ERK, I\textkappa}B\alpha, and p38MAPK, compared with non-perfused counterparts (Fig 6I). \textit{Rab11a\textsuperscript{ATEC}}-germ-free intestines showed higher levels of ERK phosphorylation, confirming an acute and stronger activation of MAPK pathway in IECs in the absence of Rab11a. Luminal perfusion of TLR9 agonists failed to activate cytokine genes (IL6 and CXCL1) in wild-type germ-free mice (Fig 6J), but drastically activated IL6 and CXCL1 levels (Fig 6I) in \textit{Rab11a\textsuperscript{ATEC}}-germ-free mice, suggesting that in the absence of Rab11a, IECs could not tolerate apical TLR9 ligand loading. Both wild-type and \textit{Rab11a\textsuperscript{ATEC}}-germ-free intestines responded to LPS perfusion, whereas the latter showed much more pronounced cytokine responses (Fig 6J), consistent with previous reports that the immune-suppressive function of apical TLR9 plays a role in dampening other TLR agonist stimulations (Lee \textit{et al}, 2006). Based on these data, we proposed that Rab11a deficiency impaired IEC's tolerance to microbial TLR agonists.

\section*{Discussion}

Our studies provided genetic evidence for the contribution of Rab11 to intestinal host-microbial homeostasis. Using cell type-specific inducible gene ablation, we demonstrated that Rab11-deficient mouse and \textit{Drosophila} enterocytes activated inflammatory signaling pathways and overproduced cytokines, causing IBD phenotypes in both species. Luminal perfusion of germ-free live mouse intestines with distinct microbial TLR agonists demonstrated that Rab11 deficiency broke the mucosal tolerance to TLR agonists. These data suggested an evolutionarily conserved function of Rab11 endosomal compartment in control of intestinal host-microbial interaction.

The endosomal gene network associated with Rab11-mediated trafficking activity is upregulated during terminal differentiation of the mouse intestinal epithelial cells (Gao \& Kaestner, 2010). Expression and functional activation of this important membrane trafficking process may reflect the increased demand by the postnatal IECs that encounter and adapt to the large microbial population after birth. At steady-state conditions in wild-type mice, the direct and constitutive recruitment and sorting of microbial sensors by Rab11a endosome may serve another important immune regulatory function dampening microbial receptor induced immune response to normal microbiota (Fig 7A). Rab11a was reported to influence cell junction in \textit{vitro} (Wang \textit{et al}, 2000; Desclouzeaux \textit{et al}, 2008), therefore, we initially suspected that the epithelial leakiness in these mutant mouse intestines might be an enteritis-triggering factor (Su \textit{et al}, 2009); however, this hypothesis was ruled out by barrier function tests. Somewhat surprisingly, \textit{Rab11a\textsuperscript{ATEC}} intestines exhibited even stronger barrier function than wild-type littermates. In fact, loss of Rab25, a member of the Rab11 subfamily also increased Claudin-1 expression and trans-epithelial resistance (Krishnan \textit{et al}, 2013), consistent with our data suggesting that the epithelial barrier function in \textit{Rab11a\textsuperscript{ATEC}} intestine is not impaired.

In cultured human IECs, apical versus basolateral compartmentalization of TLR9 has been linked to the distinct immune responses elicited by ligand-activated TLR9 receptors (Lee \textit{et al}, 2006). Indeed, in our perfusion assays, wild-type germ-free mouse intestines were largely tolerant to luminal loading of purified TLR9 agonists, as no transcriptional activation of IL6 and CXCL1 was detected. This tolerance appeared to be TLR9-specific, since luminal LPS loading activated both genes in wild-type intestines. However, in the absence of Rab11a, luminal loading of TLR9 agonists dramatically induced IL6 and CXCL1 expression, suggesting that in wild-type IECs, the Rab11a vesicles suppressed TLR9 activation. Since TLR9 activation from the apical surface induced IEC tolerance to subsequent TLR agonist stimulation (Lee \textit{et al}, 2006), complete loss of this protective mechanism in Rab11a-deficient IECs may be reflected by the strong cytokine response induced by LPS. Alternatively, Rab11a has been reported to recruit TLR4 to phagosomes activating type I interferon response in human monocytes (Husebye \textit{et al}, 2010). We, at this moment, could not exclude the possibility of pro-inflammatory TLR4 signaling due to missorting of TLR4 in wild-type intestines exhibited strong phosphorylation of Erk in wild-type intestines, we speculated that Rab11a sorting vesicles might be essential to control the strength of receptor signaling.

Endolysosomal control of TLR9 proteolysis and activation has been described in innate immune cells, but has not been well explored in IECs. Adult Rab11a-deficient IECs showed clearly altered fragmentation patterns compared to controls. In fact, independent intestines from various mutant animals, regardless of their genders, demonstrated strikingly similar fragmentation pattern to each other. This implied that, in Rab11a’s absence, TLR9 was consistently transported into a certain compartment where it was improperly processed. In macrophages, the ectodomain of TLR9 is cleaved in a process called endolysosomes derived from germ-free mice showed decreased IL-1\alpha production and reduced proliferation, compared with SPF IECs at SPF intestines, respectively. 

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
Organoid & IL-1\alpha (pg/ml) & IL-6 (pg/ml) \\
\hline
Germ-free & 1890 & 1891 \\
\hline
SPF & 1890 & 1891 \\
\hline
\end{tabular}
\caption{Growth and secretion of IL-1\alpha and IL-6 in organoids derived from germ-free and SPF intestines.}
\end{table}

However, Rab11a expression decreased more in the absence of Rab11a in both genotypes, consistent with the data suggesting that the epithelial barrier function in \textit{Rab11a\textsuperscript{ATEC}} intestine is not impaired.
Figure 6. Rab11a controls epithelial tolerance to microbial TLR agonists.

A Barrier function tests showed that Rab11a<sup>−/−</sup> epithelia were not leaky. The active accumulation of permeabilized radioactive molecules was shown as a ratio of inside/outside quantity of <sup>3</sup>H-l-glucose or <sup>14</sup>C-inulin. Rab11a<sup>−/−</sup> duodenum and ileum showed stronger barrier function than wild-type littermates. ***P < 0.001.

B, C Germ-free Rab11a<sup>−/−</sup> mice showed reduced crypt hyperplasia compared to Rab11a<sup>+/+</sup> mice at SPF condition. Scale bars, 10 μm. **P < 0.01, ***P < 0.001.

D, E Germ-free Rab11a<sup>−/−</sup> mice had decreased levels of serum IL-6 and intestinal IL6 mRNA, compared with SPF Rab11a<sup>+/+</sup> mice. *P < 0.05, **P < 0.01.

F, G Rab11a<sup>−/−</sup> organoids derived from germ-free mice showed decreased IL-6 production and reduced proliferation, compared with SPF Rab11a<sup>+/+</sup> mice. *P < 0.05, **P < 0.01, ***P < 0.001.

H Luminal perfusion assays, using specific TLR agonists (E. coli DNA or LPS), were performed on 4-week-old germ-free live animals: Rab11a<sup>−/−</sup> and wild-type littermates. After perfusion, the perfused intestinal tissues (red) and the immediately proximal non-perfused tissues (dotted black lines) were dissected for mRNA and protein analyses.

I Western blots for various signaling pathway effectors. TLR9 ligand-perfused Rab11a<sup>−/−</sup> intestines showed strong phosphorylation of ERK, compared with wild-type or non-perfused tissues.

J qRT-PCR for IL6 and CXCL1 was performed on TLR9 ligand-perfused wild-type and Rab11a<sup>−/−</sup> intestines. IL6 and CXCL1 expression levels in corresponding non-perfused tissues were used as baselines for each genotype. Data are shown as fold inductions. Note that TLR9 ligand perfusion decreased IL6 and CXCL1 expressions in wild-type intestines, whereas the same perfusion induced 128-fold and 64-fold increases of IL6 and CXCL1 levels in Rab11a<sup>−/−</sup> intestines, respectively.
mutations that bypass receptor proteolysis could be activated by self-DNA in dendritic cells causing lethal inflammation in mice (Mouchess et al., 2011). These scenarios, somewhat similar to what was observed in Rab11a-deficient IECs, suggested that intrinsic defects altering TLR9 sorting and processing could induce strong inflammatory responses. In Rab11a-deficient IECs, full activation of TLR9, still required its agonists, since complete removal of microbial agonists (germ-free condition) reduces the requirement for this Rab11a-mediated immuno-suppressive function.

Figure 7. Rab11a vesicles in enterocytes maintain proper TLR7 compartmentalization and intestinal host-microbial homeostasis.

A. At steady-state conditions, Rab11a vesicles constitutively sequester and/or recycle TLR7 to apical compartment or cell surface, dampening the receptor processing and activation in endolysosome. In the absence of Rab11a endosome, TLR7 shifts toward endolysosome promoting strong inflammatory response. Removal of microbial agonists (germ-free condition) reduces the requirement for this Rab11a-mediated immuno-suppressive function.

B. Loss of Rab11 in both mouse and Drosophila enterocytes induced non-autonomous cell division in the IESC compartment. This enterocyte-to-stem cell signaling appears to be mediated by enterocyte-originated cytokines. The similarities shared by both animal species hinted a well-conserved Rab11a-dependent innate mechanism employed by enterocytes to support the host-microbial homeostasis.

Materials and Methods

Mice

The mouse Rab11a conditional floxed allele was derived through homologous recombination in mouse embryonic stem cells at a 129 genetic background. A loxP site was inserted at 242 bp downstream of Rab11a’s second exon, while a second loxP site introduced at 210 bp downstream of fourth exon. Lgr5loxP-ires-CreERT2, Vil-Cre, Vil-CreER, Rab8a knockout mice have been described (Madison et al., 2002; el Marjou et al., 2004; Barker et al., 2007). Germ-free colonies were re-derived from plug mating females, housed, and bred in filtered air, autoclaved caging, bedding, food and water. Germ-free status was confirmed by PCR analyses for fecal 16rDNA. Data for mouse experiments were obtained from five to eight individual mice for each genotype group. All experiments were...
performed on littermates unless otherwise stated. Additional molecular and phenotypic analyses are in the Supplementary Information and were detailed previously (Gao et al., 2009; Gao & Kaestner, 2010; Sakamori et al., 2012).

**Drosophila**

All *Drosophila* stocks were maintained at room temperature in yeast extract/cornmeal/molasses/agar food medium. w118 were used as the wild-type for crossing with various Ga4 lines for control experiments. The fly stocks Rab11^778^ was originated from Transgenic RNAi Project of Harvard Medical School and obtained from Bloomington (#27730). The Rab11^22194^ and Rab11^2108392^ were obtained from Vienna Drosophila RNAi Center. The Ga4 drivers have been described previously (Micchelli & Perrimon, 2006; Zeng et al., 2010; Jiang et al., 2011). The Upd3-LacZ was as described (Zhou et al., 2013). Control flies were crosses between w and the Ga4 lines as indicated. All fly crosses also contained the tubulin-Gal80Δ for temporal control, and 5– to 7-day-old flies were shifted to 29°C for 4 days to allow Ga4 activity and RNAi.

**Mouse intestinal crypt isolation and organoid culture**

Procedures for crypt isolation and organoid culture were essentially the same as reported (Sato et al., 2009). Crypts were counted, resuspended in Matrigel (BD Biosciences, #354234), and seeded into 8-well chamber slides, 48-well or 24-well culture plates, depending on the purpose of the study. After Matrigel polymerization, crypt culture medium (Advanced DMEM/F12 containing 50 ng/ml recombinant murine EGF, 1 μg/ml recombinant murine R-Spondin 1, 100 ng/ml recombinant murine Noggin, 1× N2 supplement, 1× B27 supplement, 1 mM N-acetylcysteine, 2 mM GlutaMax, 10 mM HEPES, and 100 U/ml penicillin/100 mg/ml streptomycin) was applied. For in vitro treatment of organoids, 200 crypts/wells were seeded in replicates for each genotype. After formation of control and mutant organoids, fresh medium was replaced containing recombinant mouse IL-6 (100 ng/ml), IL-6 neutralizing antibody (1 μg/ml), BAY11-0782 (10 μM), or no supplement. After 24 h, the cultures were used either for cytokine ELISA or for immunofluorescent analyses detailed in Supplementary Information. All assays were independently repeated at least three times.

**Histopathology scoring of intestinal inflammation**

To assess the inflammation of small intestine, a histological scoring system as described previously (Adolph et al., 2013) was conducted on operator blinded histology sections. Briefly, this histological scoring system comprised five histological features which were evaluated semi-quantitatively (0, absent; 1, mild; 2, moderate; 3, severe): mononuclear cell infiltrate (0–3), crypt hyperplasia (0–3), epithelial injury/erosion (0–3), polymorphonuclear cell infiltrates (0–3), and transmural inflammation (0, absent; 1, submucosal; 2, one focus extending into muscularis and serosa; 3, up to five foci extending into muscularis and serosa; 4, diffuse). In addition, one extended factor was derived based on the fraction of bowel length involved by inflammation: (i) < 10%; (ii) 10–25%; (iii) 25–50%; and (iv) > 50%. The histological score was calculated as a sum of five independent parameters multiplied by the extended factor.

**Sucrose density gradient centrifugation**

Procedures were modified from Yao et al. (2009). Small intestinal tissues were suspended in cold detergent-free lysis buffer (100 mM sodium carbonate pH 11, 0.5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride and 2 μg/ml protease inhibitor (Roche Diagnostics) and lysed with 50 strokes using tight pestle in glass homogenizer. Homogenates were centrifuged at 3,000 g for 10 min at 4°C to remove nuclei, large cell debris and unbroken cells. The supernatants were adjusted to 50% sucrose by mixing with 90% (w/v) sucrose solution (in 25 mM 4-Morpholineethanesulfonic acid, 150 mM NaCl, 250 mM NaCO3), and loaded to the bottom of 12.5-ml ultracentrifugation tube. Eleven discontinuous sucrose gradients (40, 35, 25, 22.5, 20, 17.5, 15, 12.5, 10 and 5%, and homogenizing buffer) were sequentially layered on top of the lysates. After centrifugation at 100,000 g for 16 h in SW40Ti swinging-bucket rotor (Beckman) at 4°C, 12 fractions, 1 ml of each layer, were collected and stored at −80°C. Twenty microlitre of each fraction as well as total tissue lysates were denatured and subjected to Western blots.

**Intraluminal perfusion assay**

To examine the response of small intestine to specific TLR9 agonist in vivo, mice were anesthetized intraperitoneally with 60 mg/kg ketamine and 5 mg/kg xylazine initially, and subjected to midline laparotomy for exposure of the entire small intestine with intact blood vessels and nerve connections. Two small incisions were made at the proximal end of jejunum and the distal end of ileum, respectively. Catheters were inserted, secured with surgical thread, and connected to an inflow polyethylene tube. After the contents were flushed, the small intestine was continuously perfused from jejunum to ileum with KRBI buffer containing 8 μg/ml of *E. coli* ssDNA (Invivogen, Catalog #111-ssc) or 2 μg/ml of LPS (Sigma, Catalog #L4391) at a rate of 15 ml/h using a peristaltic pump for 4 h and then harvested for further analysis. The temperature of mouse body and perfusion solution was maintained at 37°C by heating pads/heat lamps and water bath, respectively.

**Statistical analysis**

Data are presented as mean values of 3–6 independently replicated experiments, with error bars representing standard error of the mean (SEM). A two-tailed Student’s t-test was used to determine significance of differences. Co-localization of dual fluorescent signals was deduced from confocal microscopic images using Pearson correlation analyses (Bolte & Cordelieres, 2006). Significance was indicated as *** when P-value < 0.05, ** P < 0.01, and **** P < 0.001.

**Supplementary information** for this article is available online: http://emboj.embopress.org

**Acknowledgements**

The authors thank Dr. Qiang Feng and Pavan Vedula for help with some graphic illustration and data analyses. This work was supported by National Institute of Health (NIH) Grants DK085194, DK093809, DK102994, and CA178599 to N.G.; DK48370 and DK070856 to J.R.G.; DK83450 to Y.T.I.; Charles
and Johanna Busch Memorial Award (659160) and Rutgers Faculty Research Grant (281708) to N.G. Y.T.I. is a member of the UMass DERC (DK22520), the UMass Center for Clinical and Translational Science (UL1TR000161) and the Guangdong Innovative Research Team Program (No. 2010001Y0104789252). S.Y. is supported by New Jersey Commission on Cancer Research Postdoctoral Fellowship (DFHS13PPC016).

Author contributions
SY, EMB, JRG, TL, and NG conceived the project, designed experiments, and analyzed data; SY, YN, BK, RS, ES, CP, SD, RPF, and VD performed experiments and analyzed data; SY, TI, and NG wrote the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

References


License: This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs 4.0 License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
**In vitro** reconstitution of a highly processive recombinant human dynein complex

Max A Schlager¹,†, Ha Thi Hoang²,†, Linas Urnavicius³, Simon L Bullock²,† & Andrew P Carter¹,***

Abstract

Cytoplasmic dynein is an approximately 1.4 MDa multi-protein complex that transports many cellular cargoes towards the minus ends of microtubules. Several *in vitro* studies of mammalian dynein have suggested that individual motors are not robustly processive, raising questions about how dynein-associated cargoes can move over long distances in cells. Here, we report the production of a fully recombinant human dynein complex from a single baculovirus in insect cells. Individual complexes very rarely show directional movement *in vitro*. However, addition of dynactin together with the N-terminal region of the cargo adaptor BICD2 (BICD2N) gives rise to unidirectional dynein movement over remarkably long distances. Single-molecule fluorescence microscopy provides evidence that BICD2N and dynactin stimulate processivity by regulating individual dynein complexes, rather than by promoting oligomerisation of the motor complex. Negative stain electron microscopy reveals the dynein–dynactin–BICD2N complex to be well ordered, with dynactin positioned approximately along the length of the dynein tail. Collectively, our results provide insight into a novel mechanism for coordinating cargo binding with long-distance motor movement.

Keywords Bicaudal-D; dynactin; dynein; microtubules; processivity

Subject Categories Membrane & Intracellular Transport

DOI 10.15252/embj.201488792 | Received 22 April 2014 | Revised 4 June 2014 | Accepted 12 June 2014 | Published online 1 July 2014

The EMBO Journal (2014) 33: 1855-1868

See also MA Cianfrocco & AE Leschniner (September 2014)

Introduction

The approximately 1.4 MDa human cytoplasmic dynein 1 complex (hereafter referred to as dynein) is the major minus end-directed microtubule motor in most eukaryotic cells (Allan, 2011; Roberts et al., 2013). It is responsible for trafficking many cellular cargoes, including organelles, vesicles and mRNAs. It is also exploited by several pathogenic viruses, which use the motor to reach specific subcellular locations (Dodding & Way, 2011). Dynein also plays fundamental roles during mitosis, with force generation of microtubule-associated motors required for breakdown of the nuclear envelope, alignment of the spindle and regulation of the spindle assembly checkpoint (Bader & Vaughan, 2010).

Dynein moves towards the minus ends of microtubules using the energy from ATP hydrolysis (Carter, 2013). The complex contains a dimer of approximately 0.5 MDa dynein heavy chains (DHCs). Each heavy chain contains a “head” region consisting of a motor domain related to the AAA+ ATPase family, which is connected to a microtubule binding domain, and a “tail” region that facilitates dimersisation and engages with smaller non-catalytic sub-units. These accessory sub-units—the intermediate chain (DIC), light intermediate chain (DLIC) and three different light chains (DLCs – Tctex, Roadblock (Robl) and LC8)—are also present in two copies per complex (King et al., 1998, 2002; Trokter et al., 2012) and have been implicated in recruitment of cargoes and regulation of motor activity. In humans, there are two genes for each accessory chain and evidence for additional spliceoforms (Pfister et al., 2006).

Despite the importance of dynein for diverse cellular functions, the mechanism by which it moves along microtubules is only partially understood. The motile behaviour of mammalian dynein has been studied using complexes purified from brain (Mallik et al., 2005; Ross et al., 2006; Miura et al., 2010; Ori-Mckenney et al., 2010; Walter et al., 2010) and tissue culture cells (ichikawa et al., 2011), as well as complexes reconstituted from individual, recombinant components (Trokter et al., 2012). Movement of individual mammalian dynein complexes has been assayed by adhering the motor to beads (King & Schroer, 2000; Mallik et al., 2005; Walter et al., 2010), labelling accessory proteins (Ross et al., 2006; Miura et al., 2010) or by GFP tagging of the motor (Trokter et al., 2012). The extent to which individual dynein complexes can take multiple successive steps without detaching from the microtubule, a behaviour termed processivity, varied in these studies. Some groups reported a subset of dyneins undergoing processive movements with an average run length of approximately 0.7–1 μm (King & Schroer, 2000; Mallik et al., 2005; Culver-Hanlon et al., 2006; Ross et al., 2006), whereas others documented substantially shorter excursions (Ori-Mckenney et al., 2010). Other studies reported no measurably processive movement (Miura et al., 2010; Trokter et al., 2012). Several of the above...
studies have frequently observed short, back-and-forth movements of dyneins (Mallik et al., 2005; Ross et al., 2006; Miura et al., 2010; Ori-McKenney et al., 2010; Trokter et al., 2012), which have been attributed to processive bidirectional movement or one-dimensional diffusion on the microtubule lattice. Strikingly, in mammalian cells, many dynein-associated cargoes move unidirectionally for several microns (Ori-McKenney et al., 2010; Rai et al., 2013; van Spronsen et al., 2013). Thus, additional factors or the association of multiple motors with a cargo appears to be required for robust transport in vivo.

Within the cellular environment, dynein can be found complexed with dynactin, an approximately 1.2 MDa multi-subunit complex (Schroer, 2004). Dynactin is required for the vast majority of dynein functions in cells (Schroer, 2004) and can bind microtubules through the p150 (DCTN1) sub-unit (Culver-Hanlon et al., 2006). Dynactin can increase the travel distance of beads associated with mammalian dynein in vitro (King & Schroer, 2000; Culver-Hanlon et al., 2006). However, because both dynein and dynactin were absorbed non-specifically to the beads in these experiments, it was not clear whether dynactin can directly affect dynein processivity by forming a complex with it or increases bead travel distance indirectly by providing an independent attachment point to the microtubule.

Here, we demonstrate that a human dynein complex can be expressed with high yield and purity from a single baculovirus construct in insect cells. We use this complex to investigate the determinants of processive movement of mammalian dynein. Our findings reveal a key role for the N-terminal region of the cargo adaptor protein BICD2 (BICD2N), which in the presence of dynactin can convert dynein from a non-processive to a highly processive motor. We provide evidence that BICD2N and dynactin stimulate long-distance movement by regulating individual dynein complexes, rather than by inducing oligomerisation of dynein complexes. Electron microscopy provides insight into the architecture of the dynein-dynactin-BICD2N complex, with dynactin associating with the dynein tail in a discrete, well-ordered structure. Collectively, our data support a model in which BICD2 allows dynein and dynactin to interact directly on cargoes to trigger long-distance transport.

Results

Production of a recombinant human dynein complex from a single baculovirus

Production of a fully recombinant mammalian dynein is highly desirable as it allows complete control of the isoform composition of purified complexes and facilitates tagging of sub-units for analysis by microscopy or biochemistry. Trokter et al. (2012) previously succeeded in producing a recombinant human dynein complex by expressing and purifying individual components and establishing a stepwise assembly protocol. Ensembles of the recombinant human dynein were active in gliding microtubules. However, individual motor complexes were not measurably processive.

In order to facilitate analysis of the mechanisms that stimulate processivity of human dynein, we set out to establish a streamlined method to produce a recombinant complex. We co-expressed genes for all six dynein subunits—DYNC1H1 (DHC), DYNC1I2 (DIC), DYNC1I12 (DLIC), DYNLT1 (Tctx), DYNLRB1 (Robl) and DYNLL1 (LC8)—from a single baculovirus in SF9 cells. (Fig 1A). These isoforms were identical to those used by Trokter et al. (2012), except we used a ubiquitously expressed DYN112 (DIC2) isoform instead of the neuronally enriched DYNC111 (DIC1) (Ha et al., 2008; Kuta et al., 2010).

The codon usage of the dynein genes was optimised for expression in SF9 cells, followed by their insertion into expression cassettes (Vijayachandran et al., 2013) containing a polyhedrin (PolH) promoter and an SV40 terminator sequence. DHC was inserted into one plasmid (pDyn1) and the non-catalytic subunits into another (pDyn2). Sequences encoding a ZZ [a tandem IgG binding domain based on S. aureus protein A (Nilsson et al., 1987)] and SNAPf moiety were added to the 5’ end of the DHC gene, producing tags on the dynein tail that permit affinity purification and covalent labelling with bright fluorophores, respectively. The plasmid backbones contain loxP sites, which allows fusion of pDyn1 and pDyn2 using Cre recombinase to create pDyn3. This larger plasmid was inserted into the EMBaC baculoviral genome by Tn7 transposition (Vijayachandran et al., 2011, 2013). The presence of all dynein genes in the resulting baculovirus, DynBan (Fig 1A), was confirmed by PCR before it was used to express full dynein complexes in insect cells.

Recombinant human dynein complexes were purified in a two-step procedure (see Materials and Methods), with subsequent SDS-PAGE analysis revealing bands for all six dynein subunits (Fig 1B). The typical dynein yield was approximately 2 mg/l of SF9 cell culture, with the complex soluble at concentrations over 10 mg/ml. Size-exclusion chromatography with multi-angle light scattering (SEC-MALS) showed that the complex eluted as a single peak with a molecular mass of approximately 1.40 MDa (Fig 1C), close to the value predicted if all six subunits are present as dimers (1.42 MDa).

In order to determine whether the recombinant dynein complex was correctly assembled, we used negative stain electron microscopy (EM) to compare its structure to that of native mammalian dynein purified from pig brains. Inspection of single particles of recombinant dynein revealed variability in the positions of the two head domains (Supplementary Fig S1). This was also observed for the endogenous pig complexes (Supplementary Fig S1), consistent with previous analysis of native mammalian dynein (Vallee et al., 1988; Amos, 1989). We observed particles with the heads stacked together, a form referred to as a phi particle (Amos, 1989), as well as positioned apart (Vallee et al., 1988; Amos, 1989). The percentage of particles forming a phi particle was variable between different EM grids, but was typically between 10 and 20%.

Multiple single particle images were aligned on the tail domain using a binary mask and classified based on the degree of inter-head separation (see Materials and Methods and Supplementary Fig S2 for details). A movie of these 2D class averages from the recombinant human dynein particles allows the range of different conformations adopted by the heads to be clearly visualised (Supplementary Movie S1).

The class averages of the pig dynein and recombinant human dynein are highly similar (Fig 2). Within each class average, the tail roughly resembles an inverted V shape when the heads are orientated at the bottom of the structure. Each arm of the inverted V consists of three distinct structural domains (Fig 2). The two copies of domain 1, which are furthest from the heads, are closely apposed within the tail in all class averages. This is also the case for the two copies of domain 2, which are located in the middle of the tail. In contrast, the two copies of domain 3, which are closest to the heads,
are in close proximity in the phi particle classes, but not in the head-separated classes. Thus, the two copies of domain 3 appear to separate when the heads separate. It is striking that the dynein tail is in close proximity in the phi particle classes, but not in the 200–300 kDa range from a gel with better low-molecular-weight separation on which bands corresponding to the different light chains can be discriminated.

C SEC-MALS of recombinant dynein. Mean observed molar mass (Obs.) and expected (Exp.) molar mass are indicated. Expected molar mass was calculated for a dimeric complex of the DHC, DIC, DLIC, Tctex, Robl and LCB chains. \( V_0 \) indicates the void volume of the column. Source data are available online for this figure.

The resulting complex contains all the expected components, is soluble and exhibits a very similar architecture to native mammalian dynein complexes.

**Individual recombinant human dynein complexes exhibit mainly static or diffusive behaviour on microtubules**

We next investigated the activity of recombinant human dynein using microtubule gliding assays. Dyneins were non-specifically adsorbed to a glass surface and incubated with fluorescent,
polarity-marked microtubules in the presence of saturating levels of ATP. TIRF microscopy revealed that all 85 microtubules examined exhibited movements with the plus end leading (Fig 3A; Supplementary Movie S2). Thus, the purified motors are capable of minus end-directed motion.

To assay the motile properties of individual dyneins, we labelled the SNAPi moiety of DHC with the fluorescent dye tetramethylrhodamine (TMR) and added the complexes to an imaging chamber containing polarity-marked microtubules bound to the glass. TMR-dynein complexes associated stably with microtubules in the presence of saturating levels of ATP, with frequent long binding events that exceeded tens of seconds (Fig 3B). The vast majority of these complexes did not exhibit unidirectional motion. Forty-two percent of all microtubule-associated dynein complexes were static, and 57% exhibited short, back-and-forth motion with no overt net directional bias at the population level (Fig 3C). These oscillatory movements appear to be diffusive in nature as they were not inhibited by vanadate (Fig 3C and Supplementary Fig S3A), which prevents ATP hydrolysis by dynein (Shimizu & Johnson, 1983). Only 1% of microtubule-associated dynein complexes exhibited exclusively minus end-directed motion in the presence of ATP (Fig 3C). In our entire study, we observed a total of 11 unidirectional, processively moving complexes of TMR-dynein alone with a mean run length of 1.3 ± 0.2 μm and mean velocity of 399 ± 91 nm/s (errors represent SEM). Collectively, our findings are broadly consistent with those of Trokter et al (2012), who found that their recombinant human GFP–dynein complex was active in ensemble microtubule gliding assays but was not processive at the single complex level.

Together, BICD2N and dynactin convert human dynein into a highly processive motor

As described above, the presence of dynactin significantly increases travel distances of beads associated with mammalian dynein in vitro (King & Schroer, 2000; Culver-Hanlon et al, 2006). To assess the influence of dynactin on individual dynein complexes, we purified native dynactin from pig brains (Supplementary Fig S4A) and mixed it in a twofold molar excess with recombinant human TMR–dynein (that is one dynein complex to two dynactin complexes). The presence of dynactin did not detectably alter the behaviour of individual complexes of dynein along immobilised microtubules (Fig 3B and C). The approximately 1% of TMR–dynein complexes (10 in total)
A  
Microtubule gliding by immobilised recombinant dynein

B  
TMR-dynein  TMR-dynein + dynactin  TMR-dynein + BICD2N  TMR-dynein + dynactin + BICD2N

C  
Static  Diffusive  Processive

D  
Mean = 499 ± 18 nm/s  
N = 331 segments (217 complexes)

E  
Mean = 5.0 ± 0.2 µm  
N = 245 runs (217 complexes)
that were unidirectional had travel distances and velocities that were not dissimilar to those observed in the absence of dynactin. The diffusive motion of a subset of dynein complexes was also not detectably changed by the presence of dynactin (Fig 3C). Even an 80-fold molar excess of dynactin to dynein was unable to modify the motile properties of the motor (Fig 3C).

In search of other mechanisms that stimulate dynein processivity, our attention turned to the Bicaudal-D2 (BICD2) protein. This is the best characterised member of a family of four BICD and BICD-related (BICDR) proteins in mammals that act as adaptors between dynein and a wide range of cargoes, including Golgi-derived vesicles, nuclei and viruses (Dienstbier & Li, 2009; Indran et al., 2010; Schlager et al., 2010). The importance of BICD2 has recently been emphasised by the association of mutations in the human gene with dominant spinal muscular atrophy (Neveling et al., 2013; Oates et al., 2013; Peeters et al., 2013). BICD2 is an 820 amino acid protein that, based on analysis of the Drosophila orthologue (Stuurman et al., 1999; Liu et al., 2013), is likely to form a predominantly coiled-coil homodimer. The N-terminus contains binding sites for dynein and dynactin (Hoogenraad et al., 2001), while the C-terminus contains binding sites for cargo-associated proteins, such as Rab6 (Matanis et al., 2002) and RanBP2 (Splinter et al., 2010). Cargo binding to the C-terminus of BICD2 appears to release an autoinhibitory interaction with the N-terminus, thereby allowing the latter region to bind the motor complex (Hoogenraad et al., 2003). Surrogate density gradient centrifugation recently demonstrated that an N-terminal fragment of mouse BICD2 (BICD2N\textsuperscript{25–400}) can promote the interaction of native mammalian dynein and dynactin complexes \textit{in vitro} by forming a triple complex (Splinter et al., 2012). Overexpression of this region of BICD2 in mammalian tissue culture cells also promotes interaction of dynein with dynactin (Hoogenraad et al., 2003; Splinter et al., 2012). We therefore wondered whether the N-terminal region of BICD2 is sufficient to stimulate dynein processivity in the presence of dynactin.

To test this hypothesis, we produced a mouse BICD2 N-terminal fragment fused to GFP (GFP–BICD2\textsuperscript{N1–400}, referred to below as BICD2N) in S9 cells (Supplementary Fig S4B). TMR–dynein motility in the presence of BICD2N was comparable to that observed for TMR–dynein alone (Fig 3B and C). In sharp contrast, addition of a mixture of BICD2N, TMR–dynein and dynactin (20 BICD2N dimers: 1 dynein complex: 2 dynactin complexes) resulted in approximately 23% of the dynein complexes exhibiting unidirectional minus end-directed movement (Fig 3B and C). In Supplementary Fig S3B and Supplementary Movie S3). Addition of vanadate to inhibit the dynein ATPase abolished these processive movements, confirming that they were dependent on ATP hydrolysis (Fig 3C and Supplementary Fig S3C). The mean velocity of unidirectional motion of TMR–dynein in the presence of BICD2N and dynactin was 499 ± 18 nm/s (Fig 3D), which is similar to values previously reported for processive dynein movement \textit{in vitro} (King & Schroer, 2000; Mallik et al., 2005; Ori-McKenney et al., 2010). Remarkably, the movements we observed were extremely processive with a mean run length of 5.0 ± 0.2 µm (Fig 3E). Runs were frequently terminated by motor complexes reaching the minus end of the microtubule, where they could be retained (Supplementary Fig S3B). Our data demonstrate that a combination of BICD2N and dynactin is sufficient to convert recombinant human dynein into a motor that travels over very long distances towards the minus ends of microtubules. To assess whether BICD2N is associated with the processive dynein complexes, we imaged the GFP and TMR signals sequentially. Despite the low intensity and rapid photobleaching of the GFP signal, we could detect BICD2N moving with the vast majority of processive TMR–dyneins (Supplementary Fig S3D). In contrast, BICD2N was rarely detected in association with non-processive dynein complexes (Supplementary Fig S3D). Thus, in the presence of dynactin, BICD2N appears to regulate dynein processivity as a component of transport complexes.

\textbf{BICD2N and dynactin can stimulate processive movement of human dynein without inducing oligomerisation of the motor complex}

We next attempted to shed light on how dynactin and BICD2N promote dynein processivity. It has previously been demonstrated that increasing the number of associated mammalian dynein complexes can stimulate long-distance movement of beads \textit{in vitro} (Mallik et al., 2005; Ross et al., 2006). This observation may reflect cooperation between individual heads within different cargo-associated dynein complexes (Mallik et al., 2013). We therefore considered the possibility that BICD2N and dynactin stimulate processive movement of recombinant human dynein by promoting oligomerisation of the motor complex.

To test this hypothesis, we labelled two pools of individual human dynein complexes with different fluorophores and mixed them in the presence of dynactin and BICD2N. Following addition of this mix to imaging chambers, the degree of oligomerisation could be assessed by counting the proportion of microtubule-bound complexes containing both fluorophores. Formation of oligomeric complexes of two or more dyneins would be expected to show at least 50% of all microtubule-associated complexes labelled with both fluorophores (Fig 4A).

During purification, dynein complexes were labelled with either TMR or Alexa467 fluorophores using the SNAP\textsuperscript{+} moiety on DHC (Supplementary Fig S5). Spectrophotometric analysis revealed that this procedure resulted in near stoichiometric labelling of each DHC monomer within the complex (see Materials and Methods). In control experiments, roughly equimolar amounts of TMR–dynein and Alexa467–dynein were added to imaging chambers in the presence of ATP. Kymographs were then used to analyse the fluorophores present in microtubule-bound puncta. Only 6 ± 0.6% of dynein puncta contained both dyes (Fig 4B), much less than the proportion expected for oligomeric complexes of two or more dyneins. It was very rare for dual colour puncta to permanently lose the signal from a single fluorophore species, indicating that photobleaching does not strongly influence our measurements. Thus, our data indicate that the vast majority of fluorescent puncta contained an individual dynein complex. The existence of dual colour puncta suggests that there is a low degree of oligomerisation of individual dynein complexes in these assay conditions.

We next combined the mixture of TMR- and A647-labelled dynein with dynactin and BICD2N, using the same ratio of total dynein to the other components employed earlier. Following addition of the protein mixture to the imaging chamber in the presence of ATP, the proportion of microtubule-bound dynein puncta that contained signals from both fluorophores was similar to that observed when labelled dyneins alone were added to chambers...
BICD2N and dynactin can induce robust processivity by regulating individual dynein complexes.

**A** Cartoon exemplifying how a mixture of dynein labelled with different fluorophores can provide insights into how BICD2N and dynactin affect the oligomeric status of the dynein complex. In the idealised example shown, an exactly 50:50 mixture of TMR-dynein and Alexa647-dynein is predicted to result in a 25:25:50 proportion of dyneins with, respectively, signals from TMR only, Alexa67 only and both fluorophores if BICD2N and dynactin induce dimerisation of dynein complexes. Induction of higher order oligomers is predicted to result in a greater proportion of dual-labelled puncta on microtubules.

**B, C** Kymograph and quantification of mean proportion of microtubule-associated dynein puncta that have signals from TMR only, Alexa67 only and both fluorophores if BICD2N and dynactin induce dimerisation of dynein complexes. Contrast of images was enhanced so that any puncta containing both dyes could be visualised readily. Example of a dual colour (white) punctum is labelled with a yellow arrowhead. Note that slightly more dynein puncta are labelled with TMR than Alexa67, presumably as a result of multiple manual handling steps in the procedure (Supplementary Fig S5). Mean values per chamber are shown, with 6 chambers from 2 independent dynein, BICD2N and dynactin preparations analysed (10–20 kymographs analysed per chamber for each condition).

**D** Quantification of mean fluorescence intensity of TMR signals from puncta of TMR-dynein that display static, diffusive and processive movements in the absence and presence of dynactin and BICD2N. Mean values per chamber are displayed, with four chambers each for dynein and for dynactin + dynactin + BICD2N (error bars show SEM). See Supplementary Fig S6 for distribution and mean fluorescence intensity of individual particles. Mean fluorescence intensity of processive TMR-dyneins in the absence of dynactin and BICD2N could not be accurately determined due to their rarity.

(Fig 4C). Thus, the presence of BICD2N and dynactin did not induce oligomerisation of a significant fraction of the dynein population. We next investigated whether the processive subset of dynein complexes were selectively oligomerised in the presence of BICD2N and dynactin. However, this was not the case. The proportion of processive dynein puncta that contained signals from both TMR and Alexa647 was also statistically indistinguishable from the proportion of dynein complexes that were dual coloured in the absence of BICD2N and dynactin (Fig 4C; Supplementary Movie S4). Although our results do not rule out dynactin and BICD2N promoting a low degree of oligomerisation of dynein, they indicate that the overall increase in dynein processivity is not dependent on a change in

© 2014 MRC Laboratory of Molecular Biology

Max A Schlager et al  Processive recombinant dynein

The EMBO Journal

Vol 33 | No 17 | 2014  1861
oligomeric status. This conclusion was corroborated by the very similar mean fluorescent intensity of the processive TMR–dyneins observed in the presence of dynactin and BICD2N compared to non-processive dynein complexes in the presence and absence of these factors (Fig 4D and Supplementary Fig S6). Collectively, our data indicate that dynactin and BICD2N can stimulate processive movement of individual dynein complexes.

**BICD2N allows dynactin to form a discrete complex with the tail of dynein**

We next sought to characterise the interaction between recombinant human dynein, dynactin and BICD2N in more detail. We first performed size-exclusion chromatography with mixtures of proteins using a column capable of separating complexes with a molecular weight up to 7 MDa. Dynein and dynactin ran as separate peaks over size-exclusion chromatography (Fig 5A (black trace) and Supplementary Fig S7) and hence did not form a stable complex on their own. This is consistent with the results of previous studies (Quintyne et al., 1999; Habermann et al., 2001; Quintyne & Schroer, 2002; Splinter et al., 2012) and our observations that even an 80-fold excess of dynactin did not change the motile properties of recombinant dynein (Fig 3C). In contrast, in the presence of BICD2N, an additional peak was observed over size-exclusion chromatography that contains components expected for a dynein–dynactin–BICD2N (DDB) complex (Fig 5A (red trace) and Supplementary Fig S7). This observation confirms that recombinant human dynein, pig brain dynactin and mouse BICD2N can form a complex, consistent with earlier evidence from sucrose density centrifugation that mouse BICD2N can associate simultaneously with native dynein and dynactin purified from bovine brain (Splinter et al., 2012). Our DDB complex ran well clear of the column void volume, consistent with it being a single complex rather than a large oligomer. Interestingly, only a fraction of all dyneins were incorporated into this complex, offering a potential explanation for why only a subset of TMR-dyneins moved processively in the presence of dynactin and BICD2N in the motility assays.

We next attempted to visualise the individual DDB complexes using negative stain EM. Previous work has implicated the dynein subunit DIC and the dynactin subunits p150 and p50/dynamitin (DCTN2) in the interaction between the two complexes (reviewed in Schroer, 2004). However, it is not known whether dynactin is associated with dynein in a tight complex or as a loosely tethered structure. We analysed a sample derived from the size-exclusion chromatography peak containing the DDB complex (Fig 5A and Supplementary Fig S7). Twenty-seven percent of particles were readily identifiable as DDB complexes based on their different appearance to dynein and dynactin alone (Supplementary Fig S1). Inspection of single particles (Fig 5C and Supplementary Fig S8) revealed that these complexes have dynein heads at the base of a structure that is significantly larger than the isolated dynein tail. We refer to this structure as the DDB tail domain (Fig 5C). The DDB particles have no more than two motor heads, providing further evidence that dynactin and BICD2N do not induce processive movement of dynein by promoting its oligomerisation. The positions of the heads in the DDB complexes are variable with respect to each other, with a similar range of head-to-head variability as observed for dynein complexes alone (Fig 5C and Supplementary Fig S8).

In order to determine whether dynein and dynactin interact in an ordered manner, a single class average of all DDB particles was produced in which individual complexes were aligned on the tail domain using a binary mask (Supplementary Fig S2; see Materials and Methods). The DDB tail shows well-defined features (Fig 5D), which suggests that the interaction between the dynein tail and dynactin forms an ordered structure. A comparison of the class average of the DDB tail with the class averages of dynactin (produced from negative stain images of individual particles of the isolated complex) and the recombinant human dynein tail (Fig 5D) suggests that the long axis of dynactin lies approximately along the long axis of the dynein tail. Higher resolution information will be required to unambiguously determine the orientation of the pointed and barbed ends of dynactin (Schroer, 2004; Imai et al., 2006) within the DDB complex.

**Discussion**

We have developed a method to efficiently produce a fully recombinant human dynein complex. This approach will facilitate future studies of dynein in vitro, including those investigating the functional consequences on mutations that are associated with human neurodevelopmental and neurodegenerative diseases (Schiavo et al., 2013). In this study, we use the human dynein complex to shed light on the regulation of motor processivity. Our data reveal that, together, dynactin and BICD2N are sufficient to convert individual mammalian dyneins into highly processive motors that can walk along microtubules for distances that are comparable to those travelled by many cargoes in vivo (Ori-McKenney et al., 2010; Encalada et al., 2011; Rai et al., 2013). Intriguingly, the mean velocity we observe for processive movements of dynein in the presence of BICD2N and dynactin is substantially lower than the values reported for a subset of dynein-dependent cargos in cells (Kural et al., 2005; Ori-McKenney et al., 2010; Rai et al., 2013). Additional regulatory factors, or the cooperation of multiple cargo-associated motors, may play a role in producing these high velocities.

It was previously shown that the binding of full-length BICD2 to dynein and dynactin is strongly reduced compared to that observed for BICD2N (Hoogenraad et al., 2003). This observation led to the model that binding of cargo adaptors to the C-terminal region of BICD2 frees the N-terminal region to associate with the motor complex, a notion recently corroborated by mutating cargo binding residues in the C-terminal region of the Drosophila BICD2 orthologue (Liu et al., 2013). The ability of BICD2N to promote processive dynein motility in conjunction with dynactin may therefore constitute a mechanism to coordinate long-distance transport with the availability of cargo. Our size-exclusion chromatography analysis indicates that interactions between dynein, dynactin and BICD2N are not particularly strong. This may explain why only a quarter of dynein complexes were unidirectional in the presence of dynactin and BICD2N. Instability of the DDB complex could be advantageous in vivo by enabling individual components to be recycled following delivery of cargoes to their destination.

In addition to BICD2, mammals have a closely related BICD1 protein, with both proteins sharing at least some of the same cargos (Dienstbier & Li, 2009). The close similarity in protein sequence and cargo transport requirements for BICD2 and BICD1 makes it likely
Figure 5. Dynein, dynactin and BICD2N form a complex, with dynein and dynactin interacting in a well-ordered structure.

A Size-exclusion chromatography traces for a mixture of dynein and dynactin alone (black trace; 1 dynein complex to 2 dynactin complexes) and dynein, dynactin and BICD2N (red trace; 1 dynein complex to 2 dynactin complexes to 20 BICD2N dimers). DDB, dynein–dynactin–BICD2N complex. V₀ indicates the void volume of the column.

B SYPRO Ruby-stained SDS–PAGE gel of the pooled and concentrated fractions collected from the DDB peak in (A). In addition to dynein subunits and BICD2N, multiple bands corresponding to dynactin subunits are observed. p135 is an spliceoform of p150 (Tokito et al., 1996). Note that BICD2N has a predicted molecular mass of 72.4 kDa due to the presence of the GFP tag.

C Representative negative stain EM single particles (low-pass filtered to 30 Å) of the DDB complex and recombinant human dynein. Note the significantly larger tail domain of the DDB complex (white bracket) and the range of head positions for both complexes. Scale bar, 20 nm.

D 2D class average of the DDB tail compared to 2D class averages of dynactin and the recombinant human dynein tail. Alignment of the dynein and DDB tails was performed by applying a binary mask that excluded the flexible dynein heads to all particles (see Supplementary Fig S2 and Materials and Methods). This procedure results in the head domains appearing as a blur following removal of the mask. Dynactin structural features are labelled as follows: p, pointed end; s, shoulder/projecting arm; b, barbed end. The dashed lines allow a size comparison of the DDB tail domain to the dynein tail and dynactin alone. Dynactin appears to be positioned approximately along the length of dynein tail domain in the DDB complex. The positions of the pointed end, shoulder/projecting arm and barbed end cannot be unambiguously determined in the class average of the DDB tail. Scale bar, 20 nm.

Source data are available online for this figure.
that they act in an analogous manner to stimulate dynein processivity. This function of BICD proteins may also be evolutionarily conserved. It was recently shown using cellular extracts that an RNA element within an asymmetrically localising mRNA can activate highly processive movement of Drosophila dynein towards microtubule minus ends (Soundararajan & Bullock, 2014). Our current study reveals that a strong candidate to mediate this stimulation is the single fly BICD protein, which is known to be one of a small number of proteins recruited to the RNA element (Dix et al., 2013). It will be important to determine in the future whether other BICD family members such as BICDR proteins (Schlager et al., 2010) and unrelated cargo adaptors for dynein (Engelender et al., 1997; Horgan et al., 2010; van der Kant et al., 2013; van Sprongen et al., 2013) also regulate motor processivity by promoting the interaction with dynactin.

Interestingly, there is compelling evidence (Kardon et al., 2009) that S. cerevisiae dynein and dynactin interact without the need for accessory proteins. Thus, it seems there are differences in how dynein and dynactin complexes associate with each other in higher and lower eukaryotes. However, once bound, dynactin may regulate dynein activity in a similar manner in both yeast and mammals. Although yeast dynein is capable of robust motion in isolation, dynactin can stimulate run lengths by more than twofold (Kardon et al., 2009). As with the mammalian system, this increase in processivity is not caused by oligomerisation of dynein (Kardon et al., 2009).

It has previously been shown that multiple individual mammalian dynein motors can transport artificial cargoes over long distances in vitro (Mallik et al., 2005) and that multiple dyneins are associated with membrane-bound cargoes inside cells (Welte et al., 1998; Hendricks et al., 2012; Rai et al., 2013). Given the involvement of multiple dyneins, an important question is how activation of processivity of individual motors by dynactin contributes to cargo transport in vivo. One possibility is that the role of dynactin is most important for cargoes, such as individual proteins, that are too small to recruit multiple dyneins. However, the requirement for BICD proteins in the transport of large membrane-bound cargoes (Swan et al., 1999; Matanis et al., 2002; Larsen et al., 2008; Splinter et al., 2010; Hu et al., 2013) and the involvement of dynactin in most of dynein’s functions (Schoer, 2004) suggests that activation of processivity of individual motors is important even when multiple dynein motors are engaged with a cargo.

How might BICD2 and dynactin stimulate processivity of individual human dyneins? It has previously been suggested that the microtubule binding domain of p150 contributes to processivity by augmenting interactions with the microtubule (King & Schroer, 2000; Culver-Hanlon et al., 2006). However, this model has recently been challenged by the finding that the microtubule binding activity of dynactin is not required for its ability to stimulate dynein processivity in yeast (Kardon et al., 2009) or in Drosophila cells (Kim et al., 2007).

Our negative stain EM data suggest that there are extensive interactions between the dynein tail and dynactin within the DDB complex. This would be most consistent with a model in which dynactin, and possibly also BICD2N, allosterically activates the dynein motor. An allosteric role for the dynein tail is supported by the effects of a disease mutation in this region on the processivity of the motor (Ori-McKenney et al., 2010). Intriguingly, our EM analysis of isolated dynein (Fig 2) shows a correlation between the proximity of the dynein heads and the proximity of the two copies of domain 3 in the dynein tail. This suggests that interactions between these regions of the tail can influence positioning of the heads. Our EM analysis of the DDB complex suggests that dynactin could make interactions with domain 3. Although we did not detect a gross difference in the variability of inter-head distances in the DDB complexes compared to dynein alone, it is conceivable that the interaction of dynactin with domain 3 of the dynein tail allosterically modulates the positions or orientations of the heads and thus biases the motor into a processive conformation. We also cannot rule out regulation of dynein processivity through long-distance allosteric effects on the microtubule binding domains. Future experiments will investigate precisely how dynactin and BICD2N control dynein processivity.

Materials and Methods

Cloning and plasmid production

The following genes were codon optimised for expression in Sf9 cells and synthesised commercially (Epoch Life Science): DHC (DYNCH1, accession number NM_001376.4), DIC (DYNCL2, IC2C, AF134477), DLIC (DYNCL1L2, LIC2, NM_006141.2), Tctex (DYNLT1, Tctex1, NM_006519.2), LC8 (DYNLL1, LC8-1, NM_003746.2) and Rob1 (DYNLRB1, Rob1, NM_014813.3). The DYNCH1 gene was fused to a His-2Z-LTLT tag (Reck-Peterson et al., 2006) and inserted into pACEBac1 (Vijayachandran et al., 2013). Ligation-independent infusion (Clontech) cloning was used to seamlessly insert a SNAPf tag (New England Biolabs) to generate pDyn1. Genes for IC2C, LIC2, Tctex1, LC8 and Rob1 were assembled into pIDC (Vijayachandran et al., 2013), with each expression cassette separated by 30 bp linkers consisting of random sequence and a unique restriction site, to generate pDyn2. pDyn1 and pDyn2 were fused using an in vitro Cre reaction (New England Biolabs) to form pDyn3. The presence of all six dynein genes was verified by PCR.

The mouse Bicd2 (NM_027971.4) gene was codon optimised for Sf9 expression and synthesised commercially (Epoch Life Science). Sequence coding for the N-terminal 400 amino acids of BICD2 was amplified by PCR and cloned into pOMniBac (Vijayachandran et al., 2013) (modified to fuse a cassette encoding a His-2Z-LTLT-GFP tag to the 5′ end of the inserted gene) by infusion cloning.

For cloning purposes, we used Phusion polymerase (New England Biolabs) in the supplied high-fidelity buffer. To verify the presence of genes in plasmids or bacmids, we used Quickload Taq 2× master mix (New England Biolabs). Both were used according to the manufacturer’s guidelines in a Verity 96-well thermal cycler (Applied Biosystems).

Insect cell expression

For protein expression, 500-ml Sf9 cell suspension (at 1×10^6 cells/ml) was infected with 5 ml of p2 baculovirus (see Supplementary Information) and incubated in a 2-l rollerbottle (Corning) in an incubator shaker (Infors) at 27°C/124 rpm for 70–75 h. The cells were harvested by centrifugation at 2,250 g for 10 min at 4°C (JLA 8.1 rotor in a Avanti J26-XP centrifuge, Beckman).
Coulter), resuspended in ice-cold PBS and spun again for 10 min at 1,810 g/4°C (Eppendorf 5810R centrifuge). The supernatant was discarded, and the pellet flash frozen in liquid nitrogen and stored at −80°C.

**Recombinant dynein purification**

For purification of dynein complexes, a frozen pellet of 250-ml insect cell culture was thawed on ice and resuspended in lysis buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 1 mM DTT, 0.1 mM ATP, 10% (v/v) glycerol, 2 mM PMSF) supplemented with protease inhibitors (Complete-EDTA Free, Roche Applied Science) to a final volume of 25 ml. Cells were lysed in a 40-ml dounce-type tissue grinder (Wheaton) using 20–30 strokes. The lysate was cleared by centrifugation (504,000 g, 45 min, 4°C; Type 70 Ti Rotor, Beckman Coulter) and added to 3–5 ml pre-washed IgG Sepharose 6 FastFlow beads (GE Healthcare) in a 2.5 × 10 cm Econo-Column (Bio-Rad) and incubated on a roller for 2–6 h. After incubation, the dynein complexes bound to IgG Sepharose beads were washed with 50 ml lysis buffer and 50 ml TEV buffer (50 mM Tris–HCl pH 7.4, 148 mM KAc, 2 mM MgAc, 1 mM EGTA, 10% (v/v) glycerol, 0.1 mM ATP, 1 mM DTT). To fluorescently label the SNAPf tag, dynein coated KAc, 2 mM MgAc, 1 mM EGTA, 10% (v/v) glycerol, 0.1 mM ATP, 1 mM DTT). To fluorescently label the SNAPf tag, dynein coated beads were incubated with either SNAP-Cell TMR-Star or SNAP-Surface Alexa Fluor 647 substrate (New England Biolabs) as described below (see also Supplementary Fig S5). Subsequently, the beads were resuspended in TEV buffer (final volume 5–15 ml) with 50–100 μl TEV protease (4 mg/ml) and incubated at 4°C on a roller overnight. After TEV cleavage, the beads were removed and the protein of interest concentrated in a 100 K molecular weight cutoff concentrator (Amicon Ultracel, Merck-Millipore) to 1–5 mg/ml. TEV protease was removed by size-exclusion chromatography using a TSKgel G4000SXL column with a TSKgel SW40 guard column (TOSOH Bioscience) equilibrated in GF150 buffer (25 mM HEPES pH 7.4, 150 mM KCl, 1 mM MgCl₂, 5 mM DTT, 0.1 mM ATP) or a Superose 6 PC 3/2.30 equilibrated in GF50 buffer (25 mM HEPES pH 7.4, 50 mM KCl, 1 mM MgCl₂, 5 mM DTT, 0.1 mM ATP) using an Etan LC system (GE Healthcare). Peak fractions were collected, pooled and concentrated to 0.5–10 mg/ml using Amicon concentrators as described above. All purification steps were performed at 4°C. The purification of native pig brain dynein, dynactin and recombinant BICD2N is described in the Supplementary Information.

SDS–PAGE was performed using Novex 4–12% Bis–Tris precast gels using either MOPS or MES buffer (Life Technologies). Gels were stained with either the Coomassie-based reagent Instant Blue (Expedeon) or SYPRO Ruby (Life Technologies) and imaged using a Gel Doc XR system with Image Lab 4.0 software (Bio-Rad). Protein concentrations were measured using Quick Start Bradford dye (Bio-Rad) and an Ultrospec 2100 Pro spectrophotometer (Amersham). The proteins were flash frozen in liquid nitrogen and stored at −80°C. Dynein was frozen in the presence of approximately 10% (v/v) glycerol.

**SNAPf labelling**

SNAPf-dynein complexes bound to IgG Sepharose 6 beads were incubated with approximately 5 μM SNAP-Cell TMR-Star or approximately 5 μM SNAP-Surface Alexa Fluor 647 (New England Biolabs) at 4°C for 40 min. Prior to TEV cleavage, excess dye was washed away with TEV buffer. Following purification of dynein complexes, labelling efficiency was determined using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies) and shown to be 87–97% per dynein monomer for TMR and Alexa Fluor 647, respectively (equating to a labelling efficiency of 98.5 or 100% per dimeric dynein complex).

**Negative stain electron microscopy**

Negative staining was carried out using protein complexes at approximately 40 nM in GF150 buffer on plasma-cleaned carbon film on 400-square-mesh copper grids (Electron Microscopy Sciences). The sample was stained with 2% (w/v) uranyl acetate. Electron micrographs were recorded on a Gatan Ultrascan 1,000 XP CCD fitted to a FEI Tecnai G2 Spirit transmission electron microscope operating at 120 kV with a 26,000× nominal magnification (4 Å/pix, 30 e Å/Å) at 1.5 μm underfocus. Particle picking and image analysis were performed using RELION (Scheres, 2012). A small data set was picked manually and used to obtain initial 2D class averages by reference-free classification. These were subsequently used to autopick a complete data set. Incorrectly picked particles were removed by three successive reference-free 2D classifications to obtain 23,628, 27,313 and 16,478 particles for recombinant human dynein, native pig dynein and DDB complex data sets, respectively. 2D classification of the dynein samples aligned all the particles and produced classes in the phi particle arrangement and with heads apart. The same procedure classified 67% of particles in the DDB sample as dynactin, based on previous negative stain images of the isolated dynactin complex (Imai et al, 2006), 7% as dynein and 27% as DDB complexes because they were larger than either dynein or dynactin alone. The low percentage of identified dynein complexes may reflect undersampling by the autopicking algorithm as a DDB class average was used as a reference. The presence of DDB, dynein and dynactin complexes in the preparation indicates that there is some dissociation of DDB during the procedure.

Detailed visualisation of the tail region of dynein and DDB was achieved by performing further particle alignment with a binary mask (Supplementary Fig S2), which excluded the flexible head domains. This procedure resulted in a single classification for each of the pig dynein, recombinant human dynein and DDB complexes. The aligned images from the isolated dynein preparations were used to obtain initial 2D class averages by reference-free classification. These were subsequently used to obtain sub-classes based on head positions. It was not possible to perform this last step for the DDB data set due to an insufficient number of images.

**Flow chamber preparation and TIR microscopy**

Glass coverslips (Thickness No. 1) were washed with 3 M NaOH for 1 h, followed by one wash in piranha solution (40% (v/v) hydrogen peroxide, 60% (v/v) sulphuric acid) for 1 h and treatment with air plasma (Sputter Coater, Edwards) for 10 min. Imaging chambers were prepared from these coverslips using double-sticky tape and passivated glass slides as counter surfaces as described (Bieling et al, 2010). All microscopy was performed at 25 ± 1°C with a total internal reflection fluorescence microscope (Nikon) equipped with a 100× objective (Nikon, 1.49 NA Oil, APO TIRF).
The imaging system was equipped with the following lasers: 150 mW 488 nm, 150 mW 561 nm laser (both Coherent Sapphire) and 100 mW 641 nm (Coherent Cube). Images were acquired with a back illuminated EMCCD camera (iXon™ DU-897E, Andor, UK) controlled with µManager software (http://micro-manager.org/wiki/Micro-Manager). The size of each pixel was 105 × 105 nm.

Microtubule gliding assay

Porcine tubulins and polymerisation buffers were purchased from Cytoskeleton, Inc. GmpCp stabilized microtubules with plus ends marked by greater incorporation of HiLyte 488 were polymerised as previously described (Roostalu et al., 2011). Flow chambers were passivated by 5% (w/v) pluronic F-127 dissolved in water for 5 min, placed on an ice-cold metal block and washed with GF150 buffer. 300 nM of TMR-labelled SNAPf-dynein was flowed into the chamber and incubated for 10 min. Unbound motors were washed off with GF 150, followed by two washes with motility buffer (MB) (30 mM HEPES/KOH, 5 mM MgSO4, 1 mM DTT, 1 mM EGTA, 40 μM taxol, 1 mg/ml α-casein (Sigma), 2.5 mM ATP, pH 7.0) or MB containing 50 mM KCl. The flow chamber was allowed to warm up to room temperature and a solution injected containing polarity marked, HiLyte 488-labelled microtubules supplemented with 2.5 mM ATP and oxygen scavenging system (1.25 μM glucose oxidase, 140 nM catalase, 71 mM 2-mercaptoethanol, and 24.9 mM NaHCO3). Microtubules were immediately visualized, with images acquired at 1-s time intervals with 100 ms exposure times. Velocities of gliding microtubules were determined by manual analysis of kymographs produced with Fiji (http://fiji.sc/Fiji). Three chambers were analysed for each buffer condition and the mean gliding velocity per microtubule determined by subjecting the velocity histogram to a Gaussian fit using Prism 6 (GraphPad).

Assaying in utro motility of individual dyneins

Flow chambers were prepared as described above and incubated with 2 mg/ml biotinylated poly(L-lysine)-[g]-poly(ethylene-glycol) (PLL-PEG-biotin) (SuSoS AG) for 10 min, followed by two washes with MB. Chambers were then incubated with 2 mg/ml streptavidin (Sigma) for 5 min followed by two washes with MB. Biotinylated GmpCp-stabilised microtubules with plus ends marked by greater incorporation of HiLyte 647 were adsorbed by binding to surface-immobilised streptavidin as described (Soundararajan & Bullock, 2014).

Unless stated otherwise, TMR-dynein was incubated with dynactin and BICD2N in MB for 5 min on ice at a molar ratio of 1:2:20, which refers to 1 dynein dimer (1.42 MDA per complex):2 dynactin complexes (1.2 MDA per complex):20 BICD2N dimers (144.8 kDa per complex). The same molar ratio was used for the dual colour labelling experiments using a mix of TMR- and A647-labelled SNAP–dyneins (see Fig 4B and C, and Supplementary Fig S5). In experiments where no BICD2N was present, dynein and dynactin complexes were mixed at a molar ratio of either 1:2 or 1:80. The mix was supplemented with 2.5 mM ATP and oxygen scavenging system. In a subset of experiments, sodium orthovanadate (vanadate) (New England Biolabs) was added to the MB. Complexes were visualised with a TIR microscope at 4.2 fps (200 ms exposure plus 36 ms image acquisition), except when GFP-BICD2N and TMR-dynein signals were imaged sequentially (Supplementary Fig S3D). Here, complexes were visualised at 3.1 fps (200 ms exposure plus 119 ms acquisition) for each channel.

Quantification and analysis of TMR–dynein motion and fluorescence intensity

Kymographs of dynein complexes on microtubules were produced with Fiji software and the population of processive, static and diffusive complexes counted manually. Only complexes that associated with a microtubule for ≥ 1.2 s (five pixels on y-axis) were analysed. The following criteria were applied to classify complexes into the three different populations: processive—complexes showing unidirectional, minus end-directed runs for ≥ 525 nm (5 pixels on x-axis); static—no measurable motion in either plus or minus direction; diffusive—bidirectional motion with at least one excursion ≥ 525 nm. Complexes associated with microtubules for < 1.2 s and moving for < 525 nm were excluded from the analysis because they could not be quantified accurately. The majority of complexes showed only one type of behaviour. Complexes showing a combination of diffusive and static behaviour were classified according to the motile behaviour that predominated over the time of image acquisition. Complexes switching from a static or diffusive behaviour to a processive state, which were not common, were counted in the processive population of complexes. For Fig 3C, mean proportions of each motile state were derived from 3 to 5 different chambers (200–300 complexes in total) per experimental condition. Run lengths and velocity were calculated from manual analysis of kymographs. A run was defined as a bout of motion of a unidirectional TMR–dynein that could be terminated by a pause or detachment from the microtubule. Twenty percent of processive runs contained bouts of motion with different velocities (constant velocity segments). Mean velocity was therefore calculated from these individual segments.

To determine fluorescent intensity of puncta of TMR–dynein, background was subtracted using the rolling ball algorithm with a ball radius of five pixels (525 nm). The fluorescence intensity per particle was determined by averaging the background subtracted values from three frames.

Statistics

Data plotting and curve fitting was performed with Prism 6 (GraphPad). Evaluations of statistical significance are described in the respective figure legend.

Supplementary information for this article is available online: http://emboj.embopress.org

Acknowledgements

We thank Drs Anna Akhmanova, Imre Berger, Chris Johnson, Olga Perisic and Sjors Scheres for reagents and advice. We are grateful to Carina Motz and other members of our laboratories for invaluable discussions. This work was funded by the Medical Research Council, UK [MC_UP_A025_1011 (AC) and U105178790 (SB)], a Wellcome Trust New Investigator Award (WT100387) and EMBO Young Investigator Award (both to AC), a Marie Curie Intra European Fellowship (MS) and a Boehringer Ingelheim Fonds PhD Fellowship (HH).
Author contributions

MS produced baculovirus constructs and expressed and purified the recombinant dynein complex and BICD2N. MS and LU performed size-exclusion chromatography and SEC-MALS. HH performed and analysed in vitro motility assays. LU purified native dynein and dynactin and performed negative stain electron microscopy. All authors contributed to design and interpretation of experiments and writing the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Dienstbier M, Li X (2003) The EMBO Journal 18452
Dienstbier M, Li X (2003) The EMBO Journal 18452
Soundararajan HC, Bullock SL (2014) The influence of dynein processivity control, MAPs, and microtubule ends on directional movement of a localising mRNA. eLife 3: e01956

License: This is an open access article under the terms of the Creative Commons Attribution 4.0 License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
EGFR controls IQGAP basolateral membrane localization and mitotic spindle orientation during epithelial morphogenesis

Inmaculada Bañón-Rodríguez, Manuel Gálvez-Santisteban, Silvia Vergarajauregui†, Minerva Bosch, Arantxa Borreguero-Pascual & Fernando Martín-Belmonte*

Abstract

Establishing the correct orientation of the mitotic spindle is an essential step in epithelial cell division in order to ensure that epithelial tubules form correctly during organ development and regeneration. While recent findings have identified some of the molecular mechanisms that underlie spindle orientation, many aspects of this process remain poorly understood. Here, we have used the 3D-MDCK model system to demonstrate a key role for a newly identified protein complex formed by IQGAP1 and the epithelial growth factor receptor (EGFR) in controlling the orientation of the mitotic spindle. IQGAP1 is a scaffolding protein that regulates many cellular pathways, from cell-cell adhesion to microtubule organization, and its localization in the basolateral membrane ensures correct spindle orientation. Through its IQ motifs, IQGAP1 binds to EGFR, which is responsible for maintaining IQGAP1 in the basolateral membrane domain. Silencing IQGAP1, or disrupting the basolateral localization of either IQGAP1 or EGFR, results in a non-polarized distribution of NuMA, mitotic spindle misorientation and defects in single lumen formation.

Keywords cell polarity; cytoskeleton; epithelial morphogenesis; lumenogenesis; spindle orientation

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Cell Cycle

DOI 10.1002/embj.201385946 | Received 11 June 2013 | Revised 11 November 2013 | Accepted 12 November 2013


Introduction

One of the most fundamental processes during development is the formation of epithelial sheets and tubes. These structures constitute the main elements in most of our internal organs (e.g. the kidney, breast or liver) and the surfaces across which an organism interacts with its environment (e.g. the skin, mucosa). In internal organs, epithelial cells usually become organized into tubules, which play essential roles in normal body functions (e.g. nutrient transport and waste disposal). During tubule formation cells first become polarized and they are then organized three-dimensionally around a central lumen, a process that is wholly dependent on the correct orientation of the mitotic spindle during cell division (Fischer et al., 2006; Rodríguez-Fraticelli et al., 2011).

During cell division, astral microtubules are guided to specific sites in the cell cortex where they anchor the mitotic spindle in the correct orientation. Epithelial cells divide along a plane of division perpendicular to the apicobasal axis, which requires the spindle to be anchored to the junctional plasma membrane. Misorientation of the spindle results in tissue and organ misshaping, and it is implicated in diseases like cancer and polycystic kidney disease (Castanon & Gonzalez-Gaitan, 2011). The αGI-LGN-NuMA protein complex controls spindle orientation by anchoring astral microtubules to the cell cortex (Siller & Doe, 2009; Hao et al., 2010). αGI localizes to the plasma membrane and recruits LGN to this region. Although αGI is distributed throughout the cell membrane in polarized tissues, LGN binds to αGI exclusively at the basolateral membrane via the activity of aPKC, which is restricted to the apical membrane where it phosphorylates LGN, thereby excluding it from this domain. However, in some epithelial cells such as chick neuroepithelial cells and Drosophila follicular epithelium, lateral distribution of LGN is independent of aPKC (Peyre et al., 2011; Bergstralh et al., 2013). In turn, αGI-LGN binds to NuMA, thereby anchoring the astral microtubules to the basolateral membrane (Blumer et al., 2006; Morin et al., 2007; Zheng et al., 2010; Peyre et al., 2011). Despite this knowledge, the precise nature of the signals that drive mitotic spindle orientation in epithelial cells is poorly understood.

The IQGAP family is an evolutionarily conserved family of multidomain proteins that are expressed in organisms from yeast to mammals. IQGAP1 is ubiquitously expressed and it is the best-characterized member of the family. IQGAP2 is mainly expressed in the liver, while IQGAP3 is restricted to the brain, lung, testis, small intestine and colon (White et al., 2012). IQGAP1 is a 190 kDa protein with an F-actin-binding calponin-homology domain (CHD), a coiled-coil homodimerization domain, a polyproline binding region (WW), an IQ...
domain with four IQ motifs, a Ras GTPase-activating protein related domain (GRD), and a RasGAP C-terminus (RGCG) that mediates binding to proteins such as E-cadherin, β-catenin, APC and CLIP-170 (Fukata et al., 2002; Johnson et al., 2009; White et al., 2009). Over 90 proteins have been described as IQGAP1 binding partners, linking IQGAP1 to several cellular functions, including cell-cell adhesion, the reorganization of actin filaments or microtubules, cell migration and the regulation of signal transduction (White et al., 2012). Indeed, the angiogenesis and metastasis seen in different types of tumors is frequently correlated with altered IQGAP1 distribution and protein levels (Johnson et al., 2009). Furthermore, IQGAP1 appears to be involved in the microtubule dynamics associated with cell polarization and silencing IQGAP1 expression inhibits the reorientation of the microtubule-organizing centre (MTOC) in several cell types (Watanabe et al., 2004; Kanwar & Wilkins, 2011). In addition, IQGAP1 is known to mediate the capture and stabilization of microtubules at the cell cortex through its binding partner CLIP170, which is essential for protein polarization and directional cell migration (Noritake et al., 2005). However, while IQGAP1 anchors microtubules to the cell cortex in a variety of cell models, its role in anchoring astral microtubules to the plasma membrane during epithelial cell division has not yet been evaluated.

Previous studies of epithelial cells revealed a positive role for IQGAP1 in E-cadherin-mediated cell-cell adhesion (Noritake et al., 2004). However, the role of IQGAP1 in other aspects of epithelial morphogenesis, such as lumen formation, remains unclear. Interestingly, IQGAP1 binds to the EGFR via its IQ motifs (McNulty et al., 2011) and this receptor has recently been shown to localize to the basolateral membrane in MDCK cysts in 3D cultures. Furthermore, this distribution of EGFR appears to be essential for single lumen formation, as mutations that drive the EGFR to the apical membrane severely affect cyst formation (Cotton et al., 2013).

Using the organotypic 3D-MDCK cell system, we describe here a new mechanism whereby the EGFR mediates the distribution of IQGAP1 in the membrane and thereby regulates mitotic spindle orientation during epithelial morphogenesis. We demonstrate that IQGAP1 localizes to the basolateral membrane to correctly orient the mitotic spindle and that the IQ motifs in IQGAP1, which are essential for its efficient binding to the EGFR, determine its specific basolateral distribution. Disrupting the localization of EGFR to the basolateral membrane or IQGAP1 binding to EGFR interferes with the restricted distribution of IQGAP1 in this domain, provoking misorientation of the mitotic spindle and defects in single lumen formation. Furthermore, IQGAP1 is required for the basolaterally restricted distribution of NuMA but not that of LGN, the localization of which is independent of IQGAP1.

Results

IQGAP1 orientates the mitotic spindle to control single lumen formation

IQGAP1 is an effector of Rac1 and Cdc42 (Briggs & Sacks, 2003) that is expressed ubiquitously in different polarized tissues of vertebrates, playing a central role in cell-cell adhesion, cell polarization, directional cell migration and the regulation of protein trafficking (Noritake et al., 2005; Osman, 2010). To define how IQGAP1 participates in epithelial lumen formation during morphogenesis, we silenced endogenous IQGAP1 and investigated its role in 3D epithelial morphogenesis in the 3D-MDCK model system (Fig 1A). A siRNA heteroduplex (#2) was seen to decrease endogenous IQGAP1 levels by 90% and therefore, it was used in the subsequent experiments (Fig 1B). When IQGAP1 expression was silenced in this way, a significant number of MDCK cysts displayed defects in single lumen formation when compared to the control cysts that formed (Fig 1C,D). Since this effect was rescued by expressing a siRNA-resistant mouse IQGAP1 isoform (described below in Fig 3), it would appear that IQGAP1 is required for correct lumen formation during 3D epithelial morphogenesis.

It was recently demonstrated, both in vivo and in 3D cultures, that the formation of single lumens is dependent on the correct orientation of the mitotic spindle during cell division (Jaffe et al., 2008; Hao et al., 2010; Rodriguez-Fraticelli et al., 2010). Indeed, disrupting any component of the machinery involved in spindle orientation affects single lumen formation. For lumen formation, the mitotic spindle is correctly orientated perpendicular to the apical basal cell axis when the astral microtubules of the spindle are captured and attached to the lateral membrane of polarized epithelial cells. Since IQGAP1 captures and stabilizes microtubules at the cell cortex (Noritake et al., 2005) we investigated the specific role of IQGAP1 in spindle orientation in the 3D-MDCK model system. In the absence of IQGAP1, misorientation of the mitotic spindle could be detected by immunofluorescence (Fig 1E), and whereas the spindle was mainly oriented perpendicular to the apical basal axis in control cysts, in IQGAP1-silenced cysts the mitotic spindles appeared to be randomly arranged (Fig 1F). Moreover, silencing IQGAP1 using different siRNAs produced similar effects on mitotic spindle orientation (supplementary Fig S1).

In addition to controlling spindle orientation, vectorial transport to a nascent apical domain is one of the molecular processes required for the formation of biological tubes (Lubarsky & Krasnow, 2003). Each cell in the cyst must coordinate its vectorial membrane transport in order to form a single lumen (Bryant et al., 2010; Galvez-Santisteban et al., 2012). As IQGAP1 is also involved in exocytosis and vesicle trafficking (Osman, 2010; White et al., 2012), and single lumen formation is defective in its absence, we investigated whether defects in protein trafficking contribute to the phenotype observed following IQGAP1 silencing. To investigate the possible role of IQGAP1 in vesicle trafficking, we silenced IQGAP1 and as a positive control, we also silenced the synaptotagmin-like protein 2a (Slp2a) that is known to play an essential role in membrane trafficking during lumen formation (Galvez-Santisteban et al., 2012). Similar defects in single lumen formation were observed in both Slp2a- and IQGAP1-silenced cells (supplementary Fig S2A), although most Slp2-silenced cells displayed defects in vesicle trafficking (the accumulation of internal vesicles) that were not observed in IQGAP1-silenced cells (supplementary Fig S2A). The failure of IQGAP1 to affect vesicle trafficking was also evident through the apical recovery of podocalyxin, which was unaffected in biotinylation assays in the absence of IQGAP1 (supplementary Fig S2B). Finally, we performed a calcium switch experiment to analyze the recovery of transepithelial resistance (TER) in vivo using an electrical cell-substrate impedance system (ECIS; Lo et al., 1995). In this way we demonstrated that IQGAP1 does not regulate the formation of apical junctions (supplementary Fig S2C) and moreover, we found that IQGAP1 does not control the position of centrosomes (supplementary Fig S3).

Since it was previously demonstrated that spindle misorientation induces multilumen formation, then defects in cystogenesis would be blocked by inhibiting cell division (Zheng et al., 2010). To further
The angle formed between the mitotic spindle and the apicobasal axis was measured and the mean angles were: siControl = 1.

Figure 1. IQGAP1 is necessary for correct spindle orientation and single lumen formation.

A MDCK cells were transfected with three different siRNA heteroduplexes targeting canine IQGAP1. Transfected cells were grown in 3D cultures for 72 h and the efficiency of the siRNAs was analyzed in Western blots (WB).

B Quantification of IQGAP1 depletion. The siRNA#2 was most effective in silencing IQGAP1, resulting in 90% depletion, and it was therefore used in all the subsequent experiments (n = 3, values represent mean ± s.d.).

C MDCK cells were transfected with control or IQGAP1 siRNA and grown in 3D cultures. After 72 h the cysts were stained for Podxl (red), actin (green) and TePro3 (blue). Scale bars, 5 μm.

D Single lumen formation was quantified with respect to that observed in control cysts. Only 59.5 ± 5.75% of siRNA#2 cysts polarized and formed a single lumen (n = 3, values represent mean ± s.d.).

E Control and IQGAP1 siRNA cysts were stained for actin (red) and acetylated tubulin (green). Scale bars, 5 μm.

F The angle formed between the mitotic spindle and the apicobasal axis was measured and the mean angles were: siControl = 75.53 ± 2.69°; siIQGAP1 = 45.47 ± 4.57° (n = 3, >40 cysts per experiment, values represent mean ± s.e.m.).

G Scheme of the experimental design (left panel): Control cells and IQGAP1 KD cells were grown in 3D cultures immediately after nucleofection. After 4 days, control cells organized in epithelial organoids surrounding a central lumen (group G1), while IQGAP1-KD cysts displayed defects in single lumen formation when compared to the controls (group G3). Control cells treated with thymidine (2 mM), formed smaller cysts with single lumens in the same rate as those not treated with the mitotic inhibitor (group G2). After 48 h, IQGAP1 interference starts to be effective, although the addition of thymidine at this point completely recovered normal lumen formation (group G4). Right panel) Representative images of cysts grown for 4 days under the conditions described previously. Scale bars, 5 μm.

H Single lumen quantification under each condition described in (G). Cysts forming single-lumen: group G1 = 73.66 ± 1.23%; group G2 = 71.36 ± 7.84%; group G3 = 52.16 ± 5.13%; group G4 = 69.98 ± 6.19% (n = 3, >100 cysts per experiment; values represent mean ± s.d.). Error bars represent the s.e.m. or s.d.: *P < 0.05; **P < 0.01.

Source data are available online for this figure.
confirm the role of IQGAP1-silencing in single lumen formation through the control of spindle orientation, we analyzed how IQGAP1 silencing affected cells maintained in the presence or absence of the mitotic inhibitor, thymidine. Importantly, we observed that when cells with silenced IQGAP1 expression were exposed to thymidine for 2 days they completely recovered normal lumen formation (Fig 1G, H), despite reducing the size of the cyst, as would be expected.

Taken together, these results indicate that IQGAP1 controls the orientation of the mitotic spindle but that it is not required for vesicle trafficking when lumens are formed during epithelial morphogenesis.

**PMA treatment induces IQGAP1 depolarization and mitotic spindle misorientation**

We next characterized the distribution of IQGAP1 to determine if its localization in 3D cysts is essential for spindle orientation. In mitotic cells, IQGAP1 was distributed throughout the basolateral membrane and it was completely absent from the apical surface (Fig 2A), while in non-mitotic cells, IQGAP1 was localized at cell-cell junctions, as observed previously in epithelial monolayers (Fig 2B; Katata et al, 2003). We assessed how altering the distribution of IQGAP1 affected mitotic spindle orientation. Stimulating gastric glands with PMA, a potent PKC activator, promotes the redistribution of IQGAP1 to the apical pole of cells lining the gland lumen (Chew et al, 2005). In MDCK cysts treated with PMA (4 nM), we observed a rapid translocation (15 min) of endogenous IQGAP1 from the basolateral to the apical surface, while the distribution of the cell-cell junction protein β-catenin remained unaffected (Fig 2C). Relocalization of IQGAP1 to the apical domain upon exposure to PMA was accompanied by a significant decrease in the mean spindle angle in MDCK cells (Fig 2D,E). These data suggest that the spatial restriction of IQGAP1 to the basolateral membrane is essential for proper spindle orientation, and that translocation of IQGAP1 to the apical membrane underlies the spindle misorientation.

PMA induces robust phosphorylation of human IQGAP1, which in turn promotes neurite outgrowth (Li et al, 2005), with Ser1443 and Ser1441 identified as the major sites of IQGAP1 phosphorylation following PMA treatment (Li et al, 2005). However, the alignment of human IQGAP1 with homologues from other species (note that MDCK cells are canine kidney cells) revealed that Ser1441 but not Ser1443 is conserved (supplementary Fig S4A). To study the role of PMA-mediated IQGAP1 phosphorylation in the apical translocation of IQGAP1, we expressed a phospho-competent S1441A mutant that localizes to the junctions of 3D-MDCK cells. Upon exposure to PMA, the IQGAP1-S1441A mutant translocated to the apical membrane at the same rate as the wild-type protein (supplementary Fig S4B), indicating that the apical translocation of IQGAP1 is not driven by PMA-mediated phosphorylation of IQGAP1 at Ser-1441. Hence, an alternative mechanism must be responsible for this effect.

**IQ motifs mediate the basolateral restriction of IQGAP1**

The IQGAP1 protein contains multiple protein-protein interacting domains that have been implicated in various functions in the cell (White et al, 2012). To better characterize the role of IQGAP1 in epithelial morphogenesis and to analyze the role of these domains in IQGAP1 localization, we generated a GFP-tagged full-length IQGAP1 and a set of GFP-IQGAP1 constructs containing different regions of this protein (Fig 3A). We analyzed the distribution of these fusion proteins by immunofluorescence (IF) in 3D-MDCK cells and like the endogenous protein, IQGAP1-GFP localized to cell junctions (Fig 3B). Interestingly, neither IQGAP1:N nor IQGAP1:C were polarized to the apical and basolateral membranes, these constructs containing the N-terminal and C-terminal domains of IQGAP1, and sharing some of the IQ motifs (Fig 3B). We analyzed how expressing these constructs affected epithelial lumen formation and as expected, neither of the mutants restored the normal phenotype when endogenous IQGAP1 was silenced. Indeed, not only did cells expressing these constructs form multiple lumens with an abnormal morphology in most cysts, similar to IQGAP1 silenced cells (Fig 3C, D), but they also significantly attenuated normal lumen formation in cells expressing normal levels of endogenous IQGAP1 (Fig 3E). Moreover, in the absence of endogenous IQGAP1, neither of these constructs could correctly orientate the spindle, which appeared to be randomized in all cases (Fig 3F).

Together, these results suggest that the N-terminal and C-terminal domains of IQGAP1 drive the specific apical localization of this protein, and that this signal is overcome by a stronger signal directing IQGAP1 to the basolateral membrane. Indeed, the actin-binding N-terminal CHD domain and the Cdc42-binding GRD domain may account for the apical localization of IQGAP1:N and IQGAP1:C, respectively. However, in cells expressing both the IQGAP1:N and IQGAP1:C constructs, which only share part of the IQ motifs, a fraction of IQGAP1 was still retained at the basolateral membrane (Fig 3A,B), suggesting that the IQ motifs of IQGAP1 drive its polarized localization. Furthermore, an IQGAP1 construct lacking the IQ motifs, IQGAP1-ΔIQm-GFP, partially redistributed to the apical membrane domain (Fig 4A,B). The fraction of IQGAP1-ΔIQm-GFP maintained in the basolateral membrane is most likely due to the RGCT-domain of IQGAP1 which interacts with other basolateral proteins such as E-cadherin or β-catenin (Kuroda et al, 1998). Importantly, IQGAP1-ΔIQm-GFP expression in MDCK cells affected single lumen formation, an effect that was even more pronounced in the absence of endogenous IQGAP1 (Fig 4C) and that resulted in a dramatic misorientation of the mitotic spindle (Fig 4D, quantification in 4E). Finally, exposing MDCK cells expressing IQGAP1-ΔIQm-GFP to PMA did not affect the distribution of the protein (Fig 4F).

Taken together, these results suggest that IQ motifs specifically restrict the distribution of IQGAP1 to the basolateral membrane. In the absence of these IQ motifs, or upon exposure to PMA, IQGAP1 becomes depolarized at the cell membrane, resulting in aberrant mitotic spindle orientation and disrupting single lumen formation.

**EGFR stimulation induces IQGAP1 depolarization and mitotic spindle misorientation**

Several proteins have been shown to bind IQGAP1 via its IQ motifs, including calmodulin, MEK1/2, B-Raf and EGFR (Takahashi & Suzuki, 2006; Johnson et al, 2009; McNulty et al, 2011). The EGFR receptor (EGFR) is known to play several essential roles in kidney homeostasis and renal tubule repair (Zeng et al, 2009), and it is specifically distributed in the basolateral membranes of the kidney collecting ducts (Hobert & Carlin, 1995; He et al, 2002) and of MDCK cells (Hobert & Carlin, 1995; Dempsey et al, 1997). Membrane polarization of EGFR is necessary to control the proliferation
of epithelial tissues and interestingly, IQGAP1 was recently shown to modulate EGFR activation (McNulty et al., 2011). Thus, EGFR has emerged as a candidate to associate IQGAP1 to the basolateral membrane and therefore, we hypothesized that the phenotype resulting from IQGAP1–IQGAP1 and EGFR in 3D-MDCK cyst cultures. First, we confirmed that EGFR localizes to the basolateral membrane (Fig 5A, left panels), as described previously in MDCK monolayers (Hobert & Carlin, 1995), and we showed that the distribution of EGFR in this membrane domain was totally independent of IQGAP1 (Fig 5A, left panels), as described previously in MDCK monolayers (Hobert & Carlin, 1995). We assessed whether this mechanism might be involved in crucial physiological processes that require reorientation of the mitotic spindle and consequently, the formation of single lumens. Accordingly, we characterized the role of EGFR in the localization and function of IQGAP1. Importantly, EGF treatment clearly dampened the interaction of IQGAP1 with EGFR (supplementary Fig S5B) and the exclusive basolateral distribution of IQGAP1 was lost in EGF-treated cells (Fig 5E, quantification in 5F). Moreover, the angle formed between the mitotic spindle and the apicobasal axis was measured and the mean angles were: Control = 72.19 ± 3.59°; PMA 5 min = 44.45 ± 5.33°; PMA 10 min = 39.30 ± 5.41°; PMA 15 min = 39.46 ± 5.29° (n = 3, >30 cells per experiment; values represent the mean ± s.e.m.). Error bars represent the s.e.m. *P < 0.05; **P < 0.01. EGFR localizes to the basolateral membrane (Fig 5A, left panels), as described previously in MDCK monolayers (Hobert & Carlin, 1995), and we showed that the distribution of EGFR in this membrane domain was totally independent of IQGAP1 (Fig 5A, right panels). During cell division, EGFR was distributed throughout the basolateral membrane and it was totally excluded from the apical membrane domain, displaying the same distribution pattern as IQGAP1 in mitotic cells (Fig 5B). We confirmed that IQGAP1

confirmed that EGFR localizes to the basolateral membrane (Fig 5A, left panels), as described previously in MDCK monolayers (Hobert & Carlin, 1995), and we showed that the distribution of EGFR in this membrane domain was totally independent of IQGAP1 (Fig 5A, right panels). During cell division, EGFR was distributed throughout the basolateral membrane and it was totally excluded from the apical membrane domain, displaying the same distribution pattern as IQGAP1 in mitotic cells (Fig 5B). We confirmed that IQGAP1

of epithelial tissues and interestingly, IQGAP1 was recently shown to modulate EGFR activation (McNulty et al., 2011). Thus, EGFR has emerged as a candidate to associate IQGAP1 to the basolateral membrane and therefore, we hypothesized that the phenotype resulting from IQGAP1–IQGAP1 and EGFR in 3D-MDCK cyst cultures. First, we

confirmed that EGFR localizes to the basolateral membrane (Fig 5A, left panels), as described previously in MDCK monolayers (Hobert & Carlin, 1995), and we showed that the distribution of EGFR in this membrane domain was totally independent of IQGAP1 (Fig 5A, right panels). During cell division, EGFR was distributed throughout the basolateral membrane and it was totally excluded from the apical membrane domain, displaying the same distribution pattern as IQGAP1 in mitotic cells (Fig 5B). We confirmed that IQGAP1

of epithelial tissues and interestingly, IQGAP1 was recently shown to modulate EGFR activation (McNulty et al., 2011). Thus, EGFR has emerged as a candidate to associate IQGAP1 to the basolateral membrane and therefore, we hypothesized that the phenotype resulting from IQGAP1–IQGAP1 and EGFR in 3D-MDCK cyst cultures. First, we
IQGAP and EGFR regulate mitotic spindle orientation

Inmaculada Banón-Rodríguez et al.

IQGAP and EGFR regulate mitotic spindle orientation

IQGAP and EGFR regulate mitotic spindle orientation

IQGAP and EGFR regulate mitotic spindle orientation

Figure 3. IQ motifs are required to localize IQGAP1 to the basolateral membrane.

A Scheme showing the IQGAP1 constructs used, each of which was designed as a GFP fusion protein.

B MDCK cells stably expressing each construct were grown in 3D cultures to form cysts, which were fixed and stained for actin (red). The basolateral (arrows) and apical (arrowheads) localization of the mutant constructs is indicated.

C MDCK clones for each construct transfected together with the IQGAP1 siRNA were grown in 3D cultures, and the cysts were fixed and stained for Pdx1 (red) and actin (blue). The basolateral (arrows) and apical (arrowheads) localization of the mutant constructs is indicated.

D The ability of each construct to allow single lumen cysts to form in the absence of endogenous IQGAP1 was quantified and represented as a percentage of the total number of cells (n = 3, >50 cysts per experiment; values represent the mean ± s.d.).

E The angle formed between the mitotic spindle and the apicobasal axis was measured and the mean angles were: IQGAP1 = 71.56 ± 2.91°; IQGAP1·N = 48.45 ± 3.67°; IQGAP1·C = 48.01 ± 3.41° (n = 3, >35 cells per experiment; values represent the mean ± s.e.m.).

Data information: Error bars represent the s.e.m. or s.d.: *P < 0.05, **P < 0.01. Scale bars, 5 μm.

interacts with EGFR by co-immunoprecipitation, an interaction that was strongly attenuated in the absence of IQGAP1 IQ motifs (supplementary Fig S5A). It was previously demonstrated that the phosphorylation of EGFR controls its subcellular distribution in 3D-MDCK cultures, allowing it to modulate epithelial morphogenesis (Cotton et al, 2013). Moreover, PMA phosphorylates and transactivates EGFR in glioblastoma cells and in dermal fibroblasts (Amos et al, 2005; Fukaya et al, 2013), and we found that exposing 3D-MDCK cyst cultures to PMA induced the internalization of EGFR in vesicles independently of IQGAP1 expression (Fig 5C). As PMA activation of PKC is thought to mediate signaling through several pathways, we stimulated EGFR with EGF to specifically study the response of this receptor. The internalization of the EGFR in MDCK cysts treated with EGF (2 ng/ml) was similar to that observed following PMA treatment (Fig 5D).

Accordingly, we characterized the role of EGFR in the localization and function of IQGAP1. Importantly, EGF treatment clearly dampened the interaction of IQGAP1 with EGFR (supplementary Fig S5B) and the exclusive basolateral distribution of IQGAP1 was lost in EGF-treated cells (Fig 5E, quantification in 5F). Moreover, EGF stimulation induced significant defects in mitotic spindle orientation, similar to those observed in IQGAP1-silenced cells (Fig 5G, quantification in 5H) and when prolonged (24 h), it induced the development of multiple small lumens surrounding the preformed central lumen and the accumulation of luminal cell clusters (supplementary Fig S5C). To further demonstrate the importance of the basolateral localization of the EGFR in restricting the distribution of IQGAP1, we generated a construct in which the intracellular region of EGFR was fused to the C2A/B domains of Slp2a (IntraEGFR) that localize exclusively to the apical membrane in 3D-MDCK cells (Galvez-Santisteban et al, 2012). Expressing the C2A/B-IntraEGFR fusion protein in MDCK cells induced the partial translocation of IQGAP1 to the apical membrane (Fig 6A), disrupting single lumen formation (Fig 6B) and mitotic spindle orientation (Fig 6C, quantification in 6D). Together, these results suggest that EGFR, an IQGAP1 binding partner, is essential to restrict the distribution of IQGAP1 to the basolateral membrane, and that exposure to EGF modifies the basolateral localization of IQGAP1, the normal orientation of the mitotic spindle and consequently, the formation of single lumens. We assessed whether this mechanism might be involved in crucial physiological processes that require reorientation of the mitotic spindle, such as epithelial tubulogenesis and branching (Yu et al,
Indeed, EGFR activation has been shown to be crucial for embryonic kidney tubulogenesis in a model in vitro system (Sakurai et al., 1997). Exposing mature 3D-MDCK organoids to EGF (2 ng/ml) for 24 h induced epithelial tubulogenesis and the depolarization of IQGAP1 (supplementary Fig S6), suggesting a physiological role of IQGAP1 depolarization in mitotic spindle reorientation.

These findings indicate that the localization of IQGAP1 depends on EGFR, and that both proteins exhibit a polarized basolateral distribution in resting and mitotic cells. Stimulation of MDCK cysts with EGF induces the endocytosis of EGFR and IQGAP1 depolarization, which in turn provokes mitotic spindle reorientation.
The absence of IQGAP1 disrupts the basolateral localization of NuMA

It is known that LGN and NuMA localize to the basolateral membrane and control correct mitotic spindle orientation (Du et al., 2001; Gordon et al., 2001; Zheng et al., 2010). Hence, we investigated the potential role of IQGAP1 in the localization and/or activity of LGN/NuMA in spindle orientation. As described previously, LGN-GFP translocates to the basolateral membrane and orients spindle poles during mitosis (Du et al., 2001), and its distribution was completely independent of IQGAP1 (Fig 8A). By contrast, while NuMA localized to the basolateral membrane in control mitotic cells, it was distributed all over the cell membrane in the absence of IQGAP1 (Fig 8B). We addressed whether the distribution of IQGAP1 depends on LGN using the C-terminal domain of LGN (C-LGN), which acts as a dominant negative form of LGN, disrupting both the endogenous basolateral distribution of LGN and mitotic spindle orientation in MDCK cells (Rodriguez-Fraticelli et al., 2010). Interestingly, IQGAP1 localization was not affected by the expression of C-LGN (under the control of the Tet-off inducible promoter), even in mitotic cells with misoriented spindles (Fig 8C).

Together these results indicate that while the activity and basolateral localization of LGN and IQGAP1 do not appear to be related, the specific localization of NuMA to the basolateral membrane of mitotic cells depends on the presence of IQGAP1 in this domain.

Discussion

Although mitotic spindle orientation is of fundamental importance in epithelial morphogenesis and in the generation of polarized cells, it is a process that remains poorly understood (Castanon & Gonzalez-Gaitan, 2011). The findings presented here describe a new molecular mechanism involved in mitotic spindle orientation and that drives the formation of the central lumen during epithelial morphogenesis. We demonstrate that the IQGAP1 scaffolding protein localizes to the basolateral membrane where it serves to orientate the mitotic spindle perpendicular to the apico basal axis of the cell. Indeed, when this specific localization of IQGAP1 is compromised in the 3D-MDCK organotypic model, the mitotic spindle is misoriented and single lumen formation is disrupted. The localization of IQGAP1 to the basolateral membrane depends on its IQ motifs, which mediate IQGAP1 binding to EGFR. Indeed, EGFR appears to act as a linker to restrain IQGAP1 in the basolateral membrane, and it is distributed in the same pattern as IQGAP1 in interphase and mitotic cells. Moreover, EGFR appears to orientate the mitotic spindle, since the activation of EGFR by EGF induces its internalization, provoking a redistribution of IQGAP1 throughout the cell membrane and ultimately disrupting the orientation of the mitotic spindle (see model Fig 9).
The mean angles formed between the mitotic spindle and the apicobasal axis were: control = 74.33 ± 2.70°, EGF-treated = 43.04 ± 4.14° (n = 3, >40 cells per experiment; values represent mean ± s.e.m.).

Figure 5. EGFR mediates IQGAP1 localization to the basolateral membrane and controls spindle orientation.

A Control cysts and those transfected with IQGAP1 siRNA were stained for EGFR (green), actin (red) and β-catenin (blue). Arrows indicate the basolateral localization of EGFR in the absence of IQGAP1.

B Control cysts were stained for tubulin (green), actin (red) and EGFR (blue).

C Control and IQGAP1 siRNA-transfected cysts were grown for 72 h, exposed to 4 μM PMA and then stained for EGFR (green), actin (red) and β-catenin (blue).

D Control cysts were grown for 72 h, treated with EGF (2 ng/ml) and then stained for EGFR (green), actin (red) and β-catenin (blue).

E Control cysts were grown for 72 h, treated with EGF (2 ng/ml) and then stained for IQGAP1 (green), actin (red) and β-catenin (blue). The basolateral (arrows) and apical (arrowheads) localization of the endogenous IQGAP1 is indicated.

F After EGF treatment IQGAP1 was evenly distributed between the apical and basolateral membranes (45.66 ± 2.21% IQGAP1 at the apical membrane). Fluorescence intensity was quantified with ImageJ software and represented as a percentage (n = 3, >50 cysts per experiment; values represent the mean ± s.d.).

G Control and EGF-treated cysts were stained for tubulin (green), actin (red) and IQGAP1 (blue). The yellow line represents the mitotic spindle angle.

H The mean angles formed between the mitotic spindle and the apicobasal axis were: control = 74.33 ± 2.70°, EGF-treated = 43.04 ± 4.14° (n = 3, >40 cells per experiment; values represent mean ± s.e.m.).

Data information: Error bars represent the s.d. or s.e.m. *P < 0.05, **P < 0.01. Scale bars, 5 μm.
IQGAP1 binds to and stabilizes plus-end-associated microtubules at the cell cortex by interacting with plus-end-associated proteins, such as CLIP170 (Fukata et al., 2002; Noritake et al., 2004). IQGAP1 has also been shown to interact directly with CLASP2 (Watanabe et al., 2009), a family of proteins that bind to the plus-tip protein EB1 and that mediate the interaction between microtubules and the cell cortex (Mimori-Kiyosue et al., 2005). Taken together with the results presented here, the interaction of IQGAP1 with different microtubule-binding proteins (e.g. CLIP170 and CLASP2) would appear to regulate plus-end astral microtubules to the basolateral membrane during mitosis, correctly orientating the spindle in dividing epithelial cells as required for lumen formation. Indeed, MDCK cells stably expressing the GFP-tagged C-terminal of IQGAP1, which acts as a dominant negative form of IQGAP1 as it prevents the binding of endogenous IQGAP1 to CLIP170 microtubules (Fukata et al., 2002), displayed significant defects in single lumen formation (data not shown). Hence, IQGAP1 binding to microtubules appears to be essential for correct spindle orientation.

It was previously demonstrated that the NuMA/LGN/Gα protein complex is capable of generating pulling forces on astral MTs during mitosis (Du & Macara, 2004), acting as a specific controller of mitotic spindle orientation in epithelia (Hao et al., 2010; Zheng et al., 2010; Peyre et al., 2011). LGN (Leu-Gly-Asn repeats)/GPSM2 (G-protein signaling modulator 2) belong to a family of proteins that modulate G-protein activation and that transduce extracellular signals received by cell surface receptors into integrated cellular responses. LGN is distributed in the cytoplasm in interphase cells but it translocates to the cell cortex during mitosis by interacting with the Gαi membrane protein. There, LGN interacts with NuMA, a microtubule-binding protein that binds to cytoplasmic dynein (Merdes et al., 1996; Du et al., 2001) and that tethers the mitotic spindle to the membrane (Fig 9). Indeed, disrupting the interactions between endogenous LGN and Gαi, or LGN and NuMA, results in spindle misorientation and defective cystogenesis (Zheng et al., 2010). Interestingly, this protein complex is localized exclusively at the basolateral membrane in MDCK cells via the aPKC-mediated apical exclusion of LGN (Hao et al., 2010).

We found that EGFR-IQGAP1 forms another complex that also seems to be required for the polarized anchoring of astral microtubules during epithelial cell division and to control of spindle orientation. Our data indicate that a functional connection exists...
IQGAP and EGFR regulate mitotic spindle orientation

Figure 7. LLC-PK1 cells have a non-polarized distribution of EGFR and IQGAP1, and they consequently suffer spindle misorientation.

A, B LLC-PK1 cysts were grown for 72 h, fixed and then stained for (A) EGFR (green), actin (red) and β-catenin (blue), or (B) IQGAP1 (green), actin (red) and β-catenin (blue). The apical localization of endogenous EGFR or IQGAP1 is indicated (arrowheads).

C Percentage of cysts forming a single lumen: MDCK, 82.96 ± 3.17%; LLC-PK1, 51.86 ± 5.04% (n = 3, >100 cysts per experiment; values represent the mean ± s.d.).

D LLC-PK1 cysts were stained for acetylated tubulin (green), actin (red) and β-catenin (blue). The yellow line represents mitotic spindle angle.

E Mean spindle angles: MDCK = 74.73 ± 3.52°, LLC-PK1 = 45.37 ± 5.07° (n = 3, 30 cysts per experiment; values represent the mean ± s.e.m.).

F, G LLC-PK1 AP1B cysts were grown for 72 h, fixed and then stained for (F) EGFR (green), actin (red) and β-catenin (blue), or (G) IQGAP1 (green), actin (red) and β-catenin (blue). The basolateral localization of endogenous EGFR or IQGAP1 is indicated (arrows).

H The percentage of LLC-PK1-AP1B cysts forming a single lumen was quantified: LLC-PK1-AP1B, 61.57 ± 5.23% (n = 3, >80 cysts per experiment; values represent the mean ± s.d.).

I LLC-PK1-AP1B cysts were stained for acetylated tubulin (green), actin (red) and β-catenin (blue). The yellow line represents mitotic spindle angle.

J Mean spindle angles: LLC-PK1 = 45.29 ± 5.09°, LLC-PK1-AP1B = 61.80 ± 5.30° (n = 3, 30 cysts per experiment; values represent the mean ± s.e.m.).

Data information: Error bars represent the s.d. or s.e.m. *P < 0.05; **P < 0.01. Scale bars, 5 μm.
IQGAP and EGFR regulate mitotic spindle orientation

Inmaculada Bañón-Rodríguez et al

The distribution of NuMA but not LGN is altered in the absence of IQGAP1.

A Stable Tet-Off T23 cells expressing LGN-GFP were transfected with control or IQGAP1 siRNA. Doxycycline was removed from medium of stably expressing cells and they were immediately plated and grown in 3D cultures for 48 h. The cells were then fixed and stained for tubulin (red) and IQGAP1 (blue). The basolateral localization of the construct is indicated (arrow). Scale bars, 5 μm.

B MDCK cells were transfected with control or IQGAP1 siRNA and grown in 3D cultures. Cysts were fixed and stained for NuMA (green), tubulin (red) and IQGAP1 (blue). The basolateral (arrows) and apical (arrowheads) localization of NuMA is indicated. Scale bars, 5 μm.

C Stable Tet-Off T23 cells expressing Ct-LGN-myc were grown in 3D cultures for 72 h (left panel) or 48 h before doxycycline was removed from the medium for a further 24 h (right panel). The cells were then fixed, and stained for IQGAP1 (red) and 201 (blue). Scale bars, 5 μm.

between both complexes in terms of their influence on mitotic spindle orientation. We found that LGN binding to Gs and its specific exclusion from the apical domain is independent of IQGAP1, since IQGAP1 silencing had no effect on the basolateral localization of LGN. However, IQGAP1 is necessary for NuMA polarization to the basolateral membrane. Thus, the question arises as to how IQGAP1 modulates the distribution of NuMA? One hypothesis is that IQGAP1 could promote the binding of NuMA to LGN at the cell cortex. Thus, in the absence of IQGAP1, NuMA still maintains the ability to bind plus end MTs in mitotic cells and it could be dragged by the astral MTs through the cell cortex, interacting in the apical membrane domain with binding partners other than LGN. Indeed, it was recently shown that spindle positioning in symmetrically dividing human anaphase cells also depends on the interaction of NuMA with the cortical adaptor 4.1G/R (Kiyomitsu & Cheeseman, 2013). However, other studies recently demonstrated that NuMA’s anaphase distribution is independent of interactions with LGN and 4.1 in mitotic keratinocytes (Seldin et al., 2013). In addition, 4.1R localized to the basolateral...
membrane in gastric epithelial cells (Yang et al., 2009) and MDCK cells (our unpublished results), and its cortical interaction is independent of IQGAP1 (Ruiz-Saenz et al., 2011). Therefore, further studies will be required to better characterize the interaction of NuMA with the cell cortex and how IQGAP1 regulates the specific localization of NuMA to the basolateral membrane. Moreover, it remains to be determined whether other proteins might also be involved in controlling the orientation of the epithelial mitotic spindle.

We demonstrate here that the polarization of IQGAP1 to the basolateral membrane is dependent on its interaction with the EGFR. The EGFR is a receptor for members of the epidermal growth factor (EGF) family, and EGF binding to its extracellular domain causes receptor dimerization and tyrosine autophosphorylation, promoting cell proliferation, growth and migration (Yarden, 2001). The localization of EGFR to the basolateral membrane is controlled at multiple levels. First, PKC-dependent phosphorylation of EGFR at Thr654 mediates its basolateral membrane sorting (Cotton et al., 2013). Two atypical PKC (aPKC) isoforms (\(\lambda_1\) and \(\gamma\)) are localized to the apical membrane in 3D-cultured MDCK cells, from where they can phosphorylate and exclude LGN, thereby influencing mitotic spindle orientation (Hao et al., 2010). Although information regarding the substrate specificity of each PKC protein is limited, some peptides are thought to be specific to individual PKC isozymes. Indeed, EGFR Thr654 may be phosphorylated by aPKC as well as PKC (Nishikawa et al., 1997). We also demonstrate that fusion of an apical-targeting domain (C2A/B) to the intracellular region of EGFR localizes the construct to the apical region. However, this construct is unable to stably maintain its apical localization and it is rapidly redistributed to sub-apical vesicles. These observations suggest a possible role for aPKC in EGFR phosphorylation and apical exclusion, preventing spindle misorientation via a mechanism similar to that which mediates the apical exclusion of LGN. Alternatively, the C2A/B domain could induce the dimerization and phosphorylation of the intracellular region of EGFR, causing its internalization. AP1B directs EGFR to the basolateral membrane via the biosynthetic route (Cotton et al., 2013) and the EGFR accumulates apically in AP1B-deficient LLC-PK1 cells, as does IQGAP1 as a consequence. AP1B expression in LLC-PK1 induced a partial but not complete redistribution of EGFR to the basolateral membrane, reducing the apical accumulation of IQGAP1, and favoring perpendicular mitotic spindle orientation and single lumen formation. These findings indicate that AP1B expression is not sufficient to restrict EGFR to the basolateral membrane in LLC-PK1 cells, and that a small accumulation of EGFR at the apical membrane is enough to induce apical IQGAP1 localization, resulting in spindle misorientation.

In vivo, tubular regeneration involves EGFR and IQGAP1 that controls spindle orientation during asymmetric cell division. This process is tightly coupled to spindle orientation (Betschinger & Knoblich, 2013). The role of EGFR in IQGAP1 localization and spindle orientation is supported by the finding that the EGFR mediates the disassembly of the actin cytoskeleton on mitotic spindles, resulting in spindle misorientation (Hao et al., 2010). Two atypical PKC (aPKC) isoforms (\(\lambda_1\) and \(\gamma\)) are localized to the apical membrane in 3D-cultured MDCK cells, from where they can phosphorylate and exclude LGN, thereby influencing mitotic spindle orientation (Hao et al., 2010). Although information regarding the substrate specificity of each PKC protein is limited, some peptides are thought to be specific to individual PKC isozymes. Indeed, EGFR Thr654 may be phosphorylated by aPKC as well as PKC (Nishikawa et al., 1997). We also demonstrate that fusion of an apical-targeting domain (C2A/B) to the intracellular region of EGFR localizes the construct to the apical region. However, this construct is unable to stably maintain its apical localization and it is rapidly redistributed to sub-apical vesicles. These observations suggest a possible role for aPKC in EGFR phosphorylation and apical exclusion, preventing spindle misorientation via a mechanism similar to that which mediates the apical exclusion of LGN. Alternatively, the C2A/B domain could induce the dimerization and phosphorylation of the intracellular region of EGFR, causing its internalization. AP1B directs EGFR to the basolateral membrane via the biosynthetic route (Cotton et al., 2013) and the EGFR accumulates apically in AP1B-deficient LLC-PK1 cells, as does IQGAP1 as a consequence. AP1B expression in LLC-PK1 induced a partial but not complete redistribution of EGFR to the basolateral membrane, reducing the apical accumulation of IQGAP1, and favoring perpendicular mitotic spindle orientation and single lumen formation. These findings indicate that AP1B expression is not sufficient to restrict EGFR to the basolateral membrane in LLC-PK1 cells, and that a small accumulation of EGFR at the apical membrane is enough to induce apical IQGAP1 localization, resulting in spindle misorientation.

During the morphogenesis of an epithelial tissue, cells often organize into biological tubes, structures required for the function of many organs. Thus, tubule formation is a fundamental event in the generation of diverse tissue types during metazoan development and it depends on the activity of soluble growth factors, like EGF, that modulate essential physiological context suggests that IQGAP1 would be restricted to the basolateral membrane in normal kidney due to its binding to EGFR. By contrast, EGFR activation, following epithelial injury in the mature kidney or due to stimulation of tubule branching during morphogenesis, would depolarize the distribution of IQGAP1, promoting altered cell division, proliferation and restoring epithelial integrity.

Epithelial growth factor receptor is one of the growth factor receptors most commonly affected in cancer and altered EGFR has
been described in several tumor types, including breast, colon and lung tumors (Sainsbury et al., 1987; Bauknecht et al., 1989; Spano et al., 2005; Ding et al., 2008). Most drugs designed to treat such tumors target the signaling activity of this receptor. However, our data demonstrate a role for EGFR in IQGAP1 localization and spindle orientation, and since the latter has been shown to be disrupted in cancer (Ellenbroek et al., 2012; Marongiu et al., 2012; Martin-Belmonte & Perez-Moreno, 2012), it may be this activity of EGFR that is compromised in cancer. There is currently increasing evidence that cancer stem cells are responsible for the long-term maintenance of tumor growth (Clarke et al., 2006; Dick, 2008; Blanpain, 2013). Asymmetric division of stem/progenitor cells allows the generation of the differentiated cells necessary for tissue development and the maintenance of a stem cell pool, both essential processes during development, adult tissue homeostasis and regeneration. Failure to carry out asymmetric stem cell division promotes symmetric cell division, increasing the population of stem cells with a highly proliferative potential. During asymmetric cell division cell polarization is tightly coupled to spindle orientation (Betschinger & Knoblich, 2004; Roegers & Jan, 2004; Siller & Doe, 2009). The complex formed by Ga/LGN/NuMA, together with other proteins like the Par complex and the Rho GTPase Cdc42, has been implicated in both symmetric and asymmetric cell division (Siller & Doe, 2009; Williams et al., 2011). Accordingly, determining the role of IQGAP in asymmetric cell division will be an important challenge for the future.

In summary, our data describe a new molecular mechanism involving EGFR and IQGAP1 that controls spindle orientation in epithelial cells. We show that EGFR acts as a basolateral linker for IQGAP1, which in turn anchors astral microtubules of the mitotic spindle to the junctional/basolateral membrane, and drives the correct orientation of cell division, which is essential for single lumen formation during tubulogenesis.

Materials and Methods

Cell culture

MDCKII cells were cultured in complete MEM supplemented with 5% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-Gln (Gibco, Life Technologies, Carlsbad, CA, USA). MDCK cells stably expressing IQGAP1-GFP and the different constructs were generated by co-transfection with the lastin-resistant gene, and they were selected for 10 days with 0.5 mg/ml lastin. LLC-PK1, LLC-PK1 AP1B and 293T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-Gln. Cyst cultures were performed as described previously (Rodriguez-Fraticelli et al., 2010).

Antibodies and reagents

The primary antibodies used were: IQGAP1 (1:1000; 610612; BD Transduction, San Jose, CA, USA), EGFR (1:100; GR01; Calbiochem, Darmstadt, Germany), tubulin [YLI/2] (1:500; ab6160; Abcam, Cambridge, UK), acetylated tubulin (1:1000; T7451; Sigma-Aldrich, Seelze, Germany), GFP (1:500; A5455; Invitrogen, Carlsbad, CA, USA), β-catenin (1:1000; sc7199; Santa Cruz, Dallas, TX, USA), NuMA (1:250; ab36999; Abcam), γ-tubulin (1:500, T6557; Sigma), ZO1 (1:500, R40.76; Millipore, Darmstadt, Germany) and myc (1:1000; 9E10; Roche, Basel, Switzerland). The Podxl/gp135 antibody was a gift from the Ojakian laboratory (State University of New York Downstate Medical Center, USA). Peroxidase-conjugated donkey anti-mouse IgG and anti-rabbit IgG were used as secondary antibodies in western blots (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Alexa Fluor-conjugated secondary antibodies (Alexa Fluor 488, 555 or 647; Invitrogen) and TOPRO3 (nuclear/ DNA staining; Molecular Probes [Carlsbad, CA, USA], Invitrogen) were used for microscopy. PMA (P1585; Sigma), EGF (01-107; Millipore) and thymidine (T1895; Sigma) were used to treat the cells.

Vectors

IQGAP1, as well as, IQGAP1:C, IQGAP1:N, IQGAP1:CT and IQGAP1A1Qm, and C2A/B intraEGFR were cloned into a pEGBP-N1 vector (Takara Bio Inc, Otsu, Shiga, Japan). LGN and DN-LGN were cloned into pTRE2hyg vector (Adgene, Cambridge, MA, USA). The pcDNA6A EGFR-myc construct was a kind gift from Antonio Villalobo (IIB-CSIC, Madrid, Spain).

RNAi

Stealth siRNA duplexed targeting messenger RNA sequences of 25 nucleotides from canine IQGAP1 were purchased from Invitrogen. The sequences were submitted to the BLAST search engine to ensure targeting specificity. For siRNA transfection, MDCK cells were trypsinized and nucleofected (Lonza, Basel, Switzerland) with the siRNAs. After 24 h, the cells were resuspended and plated in 12-well plates and in coverglass chambers coated with Matrigel to grow cysts. Total cell lysates from 3D cultures were analyzed in western blots to confirm siRNA efficiency.

Immunofluorescence, microscopy and quantification

MDCK cells were fixed at different time points and stained by immunofluorescence using the primary antibodies indicated. Alexa-fluor488/555/647 conjugated anti-rabbit and anti-mouse were used as the secondary antibodies (Life technologies). Images were acquired on an LSM510 inverted confocal microscopes (Zeiss, Jena, Germany) using the ZEN software and 63X/NA1.4 oil Plan-Apochromat Objectives (Zeiss). Finally, the images were treated using ImageJ software (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

To analyze spindle orientation, cysts were stained for acetylated tubulin, and the angle formed between the spindle and the apico- basal axis was measured. At least three experiments per condition were quantified, analyzing 30 cysts/experiment. Significance was calculated using a paired, two-tailed Student’s t-test, and the P-values are indicated in each experiment.

Measurement of spindle angle

Confocal images of mitotic cells in the middle region of the cysts were collected. Using ImageJ software, a line connecting the two spindle poles was drawn, and another line was drawn from the center of the apical membrane of the cell to the midpoint of the basal membrane, defining the apicobasal axis. The angle between the two lines was measured.
Calcium switch

Cells were incubated overnight in calcium-free MEM medium supplemented with dialyzed FBS, subsequently restoring calcium conditions with complete MEM. Monolayer impedance was measured in real time to determine transepithelial permeability.

Apical biotinylation assay

Control and IQGAP1-silenced cells were grown to confluency on six well plates. The cells were treated twice for 30 min on ice with trypsin (100 µg/ml), they were then washed with ice-cold PBS and new medium was added. After 0, 30, 60 or 90 min of incubation at 37°C, the cells were extensively washed with ice-cold PBS before they were biotinylated for 20 min on ice (1 mg/ml sulfo-NHS-LC-biotin in PBS, supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ [pH 7.8]). The excess biotin was removed and quenching buffer (Tris–HCl 50 mM pH 6.8) was added for 10 min on ice. Finally, to carry out the pull-down assay the cells were lysed with RIPA buffer containing 0.2% Triton Tx-100 (50 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS) and protease inhibitors (Boehringer, Ingelheim, Germany).

Immunoprecipitation

The 293T cells were grown on p100 dishes until they reached 60–70% confluence and they were then co-transfected with EGFR myc-IQGAP1 eGFP or EGFR myc-IQGAP1 ΔIQm eGFP. Transfection with a GFP construct alone or with IQGAP1 eGFP served as the controls. In supplementary Fig S5A, EGFR was immunoprecipitated with an anti-myc antibody and IQGAP1 binding was assessed in Western blots. In supplementary Fig S5B, IQGAP1 was immunoprecipitated with an anti-GFP antibody and EGFR binding was assessed in Western blots probed with anti-myc. In both cases the input material was 1/20th of the immunoprecipitate volume. Binding was analyzed by quantifying the pixels using ImageJ software.

Supplementary information for this article is available online:
http://emboj.embopress.org

Acknowledgements

We thank Carmen M. Ruiz-Jarabo for her comments on the manuscript and members of Martin-Belmonte lab for helpful discussions. We thank Ryan Schreiner and Enrique Rodriguez-Boulan (Cornell University, NY, USA) for the gift of the LLC-PK1 AP1B cells. We thank George Djakian (SUNY Downstate Medical Center, USA) for the gift of the gp135/podocalyxin antibody. We also thank the optical and confocal microscopy service at the CBM50 (SMOC) for their help and technical support. This work was supported by grants from the Human Frontiers Science Program (HFSP-CDA 00011/2009), MICINN (BFU2011-22622 and CONSOLIDER CSD2009-00016) to F.M.B. I.B.-R is recipient of an Asociación Española Contra el Cáncer (AECC) grant. M.G-S is recipient of a FPI fellowship, from the MICINN.

Author contributions


Conflict of interest

The authors declare that they have no conflicts of interest.

References

Chew CS, Okamoto CT, Chen X, Qin HY (2005) IQGAPs are differentially expressed and regulated in polarized gastric epithelial cells. Am J Physiol Gastrointest Liver Physiol 288: C376–C387
Du Q, Stukenberg PT, Macara IG (2001) A mammalian Partner of insuscetable
binds NuMA and regulates mitotic spindle organization. Nat Cell Biol 3: 1069 – 1075
Fischer E, Legue E, Doyen A, Nato F, Nicolas JF, Torres V, Yaniv M, Pontoglio
Genet 38: 21 – 23
complex mediates basolateral targeting in polarized epithelial cells. Cell
99: 189 – 198
Fukata M, Watanabe T, Noritake J, Nakagawa M, Yamaga M, Kuroda S, 
Fukaya S, Matsui Y, Tomaru U, Kawai M, Sogo S, Bohgaki T, Atsumi T, 
Koike T, Kasahara M, Ishizu A (2013) Overexpression of 
TNF-α-converting enzyme in fibroblasts augments dermal fibrosis 
after inflammation. Lab Invest 93: 72 – 80
Galvez-Santisteban M, Rodriguez-Fraticelli AE, Bryant DM, Vergarauregui S, 
Yasuda T, Banon-Rodriguez I, Bernasconi I, Datta A, Spivak N, Young K 
et al (2012) Synaptotagmin-like proteins control the formation of a 
AP18 mediates post-endocytic recycling to the basolateral membrane. Nat 
Cell Biol 4: 605 – 609
Gesualdo L, Di Paolo S, Calabro A, Milani S, Maieron E, Ranieri E, 
Pannarale G, Schena FP (1996) Expression of epidermal growth factor and 
its receptor in normal and diseased human kidney: an immunohistochemical and in situ hybridization study. Kidney Int 49: 
656 – 665
mitosis requires microtubule anchorage at spindle poles. J Cell Biol 152: 
425 – 434
by aPKC-mediated phosphorylation of apical Pins. Curr Biol 20: 
1809 – 1818
juxtaposition domain has multiple basolateral plasma membrane localizations, including a dominant signal with a 
EGF receptor is required for basolateral localization in MDCK cells. J Cell
Physiol 162: 434 – 446
Jaffe AB, Kaji N, Durgan J, Hall A (2008) Cdc42 controls spindle orientation to 
position the apical surface during epithelial morphogenesis. J Cell Biol 183: 
625 – 633
Johnson M, Sharma M, Henderson BR (2009) IQGAP1 regulation and roles in 
Kanwar N, Wilkinson JA (2011) IQGAP1 involvement in MTOC and 
granule polarization in NK-2 cell cytotoxicity. Eur J Immunol 41: 
2763 – 2773
(2003) Involvement of nectin in the localization of IQGAP1 at the cell-adhesion sites through the actin cytoskeleton in Madin-Darby canine 
kidney cells. Oncogene 22: 2097 – 2109
Kiyoymitsu T, Cheeseman IM (2013) Cortical dynin and asymmetric 
membrane elongation coordinately position the spindle in anaphase. Cell
154: 391 – 402
Kuroda S, Fukata M, Nakagawa M, Fujii K, Nakamura T, Ookubo T, Izawa I, 
Nagase T, Nomura N, Tani H et al (1998) Role of IQGAP1, a target of the small 
GTPases Cdc42 and Rac1, in regulation of E-cadherin- mediated 
cell-cell adhesion. Science 281: 832 – 835
Li Z, McNulty DE, Marler KJ, Lim L, Hall C, Annan RS, Sacks DB (2005) IQGAP1 
promotes neurite outgrowth in a phosphorylation-dependent manner. 
J Biol Chem 280: 13871 – 13878
Lo CM, Keese CR, Giaever I (1995) Impedance analysis of MDCK cells 
measured by electric cell-substrate impedance sensing. Biophys J 69: 
2800 – 2807
biological tubes. Cell 112: 19 – 28
Marongiu F, Doratiotti S, Sini M, Serra MP, Laconi E (2012) Cancer as a disease of 
tissue pattern formation. Prog Histochem Cytochem 47: 175 – 207
Martin-Belmonte F, Perez-Moreno M (2012) Epithelial cell polarity, stem cells and 
McNulty DE, Li Z, White CD, Sacks DB, Annan RS (2011) MAPK scaffold 
IQGAP1 binds the EGF receptor and modulates its activation. J Biol Chem 
286: 15010 – 15021
Merdes A, Ramyar K, Vecho JD, Cleveland DW (1996) A complex of NuMA and 
cytoplasmic dynin is essential for mitotic spindle assembly. Cell 87: 
447 – 458
Mimori-Kiyosue Y, Grigoriev I, Lamsbergen G, Sasaki H, Matsui C, Severin F, 
bind to E1B and regulate microtubule plus-end dynamics at the cell 
Morin X, Jaouen F, Durbec P (2007) Control of planar divisions by the 
G-protein regulator LGN maintains progenitors in the chick 
neuroepithelium. Nat Neurosci 10: 1440 – 1448
Determination of the specific substrate sequence motifs of protein kinase 
Noritake J, Fukata M, Sato K, Nakagawa M, Watanabe T, Izumi N, Wang S, 
Fukata Y, Kaibuchi K (2004) Positive role of IQGAP1, an effector of Rac1, in 
actin-meshwork formation at sites of cell-cell contact. Mol Biol Cell 15: 
1065 – 1076
ScientificWorldJournal 10: 944 – 953
lateral belt of cortical LCN and NuMA guides mitotic spindle movements 
and polarize: recent advances in the molecular mechanism regulating 
Rodriguez-Fraticelli AE, Vergarauregui S, Eastburn DJ, Datta A, Alonso MA, 
195 – 205
regulates cell migration and IQGAP1 recruitment to the leading edge. J 
Cell Sci 124: 2529 – 2538


The kinesin KIF16B mediates apical transcytosis of transferrin receptor in AP-1B-deficient epithelia

Andres E Perez Bay1, Ryan Schreiner1, Francesca Mazzoni1, Jose M Carvaljal-Gonzalez1, Diego Gravotta1, Emilie Perret1, Guillermo Lehmann Mantaras1, Yuan-Shan Zhu2 and Enrique J Rodriguez-Boulan1,3,*

1Department of Ophthalmology, Margaret Dyson Vision Research Institute, Weill Cornell Medical College, New York, NY, USA; 2Department of Medicine/Endocrinology, Weill Cornell Medical College, New York, NY, USA; and 3Department of Cell and Developmental Biology, Weill Cornell Medical College, New York, NY, USA

Polarized epithelial cells take up nutrients from the blood through receptors that are endocytosed and recycle back to the basolateral plasma membrane (PM) utilizing the epithelial-specific clathrin adaptor AP-1B. Some native epithelia lack AP-1B and therefore recycle cognate basolateral receptors to the apical PM, where they carry out important functions for the host organ. Here, we report a novel transcytotic pathway employed by AP-1B-deficient epithelia to relocate AP-1B cargo, such as transferrin receptor (TfR), to the apical PM. Lack of AP-1B inhibited basolateral recycling of TfR from common recycling endosomes (CRE), the site of function of AP-1B, and promoted its transfer to apical recycling endosomes (ARE) mediated by the plus-end kinesin KIF16B and non-centrosomal microtubules, and its delivery to the apical membrane mediated by the small GTPase rab11a. Hence, our experiments suggest that the apical recycling pathway of epithelial cells is functionally equivalent to the rab11a-dependent TfR recycling pathway of non-polarized cells. They define a transcytotic pathway important for the physiology of native AP-1B-deficient epithelia and report the first microtubule motor involved in transcytosis.

Introduction

Endocytosis and recycling of plasma membrane (PM) receptors play a key role in the uptake of many critical cell nutrients and in the relay of extracellular signals (Maxfield and McGraw, 2004). In non-polarized cells (e.g., fibroblasts), most nutrient receptors (e.g., transferrin receptor (TfR) and low-density lipoprotein receptor (LDLR)) traffic through a single major recycling pathway that involves sequential internalization into peripheral sorting endosomes (SE) and juxtanuclear recycling endosomes (RE) (Hopkins, 1983; Yamashiro et al., 1984; Maxfield and McGraw, 2004). In contrast, in epithelial cells, which display polarized apical and basolateral PM domains and receive most of their nutrients and signals from the blood, most endocytic receptors reside at the basolateral PM and must be recycled back to this PM domain after endocytosis (Perret et al., 2005; Rodriguez-Boulan et al., 2005). In these cells, most nutrient receptors are sequentially internalized into peripheral basolateral sorting endosomes (BSE) and juxtanuclear common recycling endosomes (CRE), where they mix with apical receptors internalized via separate apical sorting endosomes (ASE) (Figure 1A) (Hughson and Hopkins, 1990; Golachowska et al., 2010). Whereas in non-polarized cells recycling of nutrient receptors such as TfR along the major recycling route is regulated by the small GTPase Rab11a, localization of AP-1B cargo to apical recycling compartments inaccessible to TfR along the major recycling route is regulated by the small GTPase Rab11a (Wang et al., 2000). Remarkably, in epithelial cells Rab11a resides at a more apically localized endosomal compartment inaccessible to TfR, the apical recycling endosomes (ARE) (Hunziker et al., 1990; Apodaca et al., 1994; Barroso and Sztul, 1994; Casanova et al., 1999; Golachowska et al., 2010), where it regulates instead the transcytotic route of polymeric IgA receptor (pIgR) and the biosynthetic route of a subset of apical PM proteins (Wang et al., 2000; Cresawn et al., 2007; Weisz and Rodriguez-Boulan, 2009).

Because in epithelial cells basolateral and apical endocytic receptors are mixed at CRE, they must be sorted by specific signals before they return to their respective surface. Sorting is mediated by apical and basolateral sorting signals structurally similar to those that sort them in the biosynthetic route (Rodriguez-Boulan et al., 2005; Weisz and Rodriguez-Boulan, 2009). Sorting of basolateral recycling receptors at CRE is mediated by a sorting machinery that includes clathrin and the epithelial-specific clathrin adaptor AP-1B (Folsch et al., 1999; Ohnno et al., 1999; Gan et al., 2002; Folsch et al., 2003; Gravotta et al., 2007; Deborde et al., 2008). Thus, the expression of AP-1B by epithelial cells appears to promote the incorporation of nutrient receptors to a recycling route to the PM that avoids interaction with Rab11a. AP-1B is widely expressed by epithelial cells. However, some epithelial cell lines, e.g., LLC-PK1 (Roush et al., 1998; Folsch et al., 1999) and some native epithelia, e.g., retinal pigment epithelium (RPE) and kidney proximal tubule, lack AP-1B (Diaz et al., 2009; Schreiner et al., 2010), which promotes apical expression of many basolateral proteins that are normally sorted by AP-1B (Roush et al., 1998; Folsch et al., 1999). Importantly, the apical localization of basolateral proteins has important physiological consequences for AP-1B-deficient native epithelia; for example, apical TfR in RPE is
thought to regulate iron levels in the retina (Garcia-Castineiras, 2010). Unfortunately, the mechanisms and pathways that mediate apical localization of basolateral proteins in AP-1B-deficient epithelia are not known.

Here, using ultrasensitive biochemical and microscopic recycling assays, we show that AP-1B-deficient epithelia incorporate two basolateral nutrient receptors, TfR and LDLR into an alternative rab11a-dependent recycling route

Figure 1 AP-1B KD MDCK cells display microtubule- and rab11a-dependent transcytosis of basolateral TfR. (A) Model of a WT MDCK cell displaying endosomal compartments and the endosomal itinerary of plgR, TfR and LDLR postendocytic pathways. (B) A SulfoTag-Tf assay. Tf labelled with biotin and the luminophore SulfoTag (SulfoTag-Tf) provides a signal 10× higher than 125I-Tf, allowing to detect 2 × 10⁻¹⁵ mol per 12 mm filter (the estimated amount of endogenous dog TfR present at the apical membrane of confluent MDCK cells). (C) MT mediate apical transcytosis of TfR. Values of apical transcytosis, basolateral recycling and the intracellular pool of SulfoTag-Tf (60 min) in control WT, nocodazole-treated WT, control AP-1B KD and nocodazole-treated AP-1B KD MDCK cells. (D) Dominant-negative rab11a inhibits apical transcytosis of TfR. A MDCK cell line was generated that stably expresses dominant-negative rab11a fused to monomeric Cherry fluorescent protein (mCh-DN-rab11a) under the control of a tetracycline-repressible promoter. Western blot analysis showed that expression of mCh-DN-rab11a increased four-fold by removing doxycycline for 12 h. (E) RNA levels of μ1B and GAPDH in WT, stable AP-1B KD, transient luciferase KD and transient AP-1B KD MDCK cells. (F) Values of apical transcytosis of SulfoTag-Tf (75 min) in WT and transient AP-1B KD MDCK cells with or without 12-h expression of mCh-DN-rab11a. *P<0.05, **P<0.001. Bars represent mean ± standard error. Ap, apical; BL, basolateral; IC, intracellular.
to the apical membrane that, like the transcytotic pathway of pIgR, involves microtubule (MT)-mediated trafficking from CRE to ARE. This pathway requires non-centrosomal MTs and the plus-end kinesin KIF16B. The requirement for this kinesin is specific, as it is not shared by pIgR. Other experiments show that expression of AP-1B in fibroblastic CHO cells generates a recycling pathway for TfR independent of rab11a. In summary, our results illustrate for the first time the case of a cargo protein (TIR) that can use both the rab11a-dependent general recycling route of non-polarized cells and the rab11a-dependent apical recycling route of epithelial cells, directly supporting the concept that the apical recycling pathway of epithelial cells is equivalent to the general recycling route of non-polarized cells. They demonstrate that the epithelial-specific adaptor AP-1B can generate signal-dependent recycling routes for TIR in both polarized and non-polarized cells and report the first MT motor involved in transcytosis across epithelia.

Results

**Ultrasensitive assay to measure TfR recycling and transcytosis**

Most studies on TfR recycling and transcytosis with $^{125}\text{I}$-Tf (transferrin) in MDCK cells were carried out overexpressing the human TfR because of the low amount of Tf transcytosed to the apical PM (Odorizzi and Trowbridge, 1997; Brown et al., 2000; Gravotta et al., 2007). Indeed, apical transcytosis of endogenous TfR requires long incubation times (i.e., 4–6 h) to allow accumulation of $^{125}\text{I}$-Tf in the apical medium (Fuller and Simons, 1986). To stay as close as possible to the physiological situation, we carried out our studies focusing on the endogenous (dog) TfR. Hence, we developed an ultrasensitive method to measure TfR recycling, that utilizes as a ligand dog Tf double-labelled with biotin and the luminophore SulfoTag (SulfoTag-Tf). This method proved to be 10 times more sensitive than $^{125}\text{I}$-Tf (Supplementary Figure S1A,B and Materials and methods). Titration curves of SulfoTag-Tf and $^{125}\text{I}$-Tf showed that only the former can detect 2 x $10^{-15}$ mol of Tf, which is the estimated amount of apical TfR present in confluent MDCK monolayers grown on a 12-mm Transwell filter (Figure 1B) (Fuller and Simons, 1986).

Using this method, we showed that wild-type (WT) MDCK cells recycle most of the basolaterally internalized SulfoTag-Tf to the basolateral membrane (85%) and transcytose a small amount of SulfoTag-Tf to the apical PM (4%) (Figure 1C). MDCK cells lacking AP-1B by stable knockdown of its medium subunit μ1B (AP-1B KD), which exhibit normal levels of transepithelial electrical resistance (Gravotta et al., 2007), display 4 x higher apical transcytosis of basolaterally internalized SulfoTag-Tf (16% versus 4%, $P < 0.001$) (Figure 1C, left) and lower basolateral recycling (71% versus 85%, $P < 0.001$), compared to WT MDCK cells (Figure 1C, middle) (see recycling kinetics in Supplementary Figure S1C).

**Apical transcytosis of TfR is microtubule- and rab11a-dependent**

MTs play important roles in apical biosynthetic trafficking of PM proteins (Rodriguez-Boulan et al., 2005; Weisz and Rodriguez-Boulan, 2009) and transcytosis of pIgR (Hunziker et al., 1990; Apodaca et al., 1994). Hence, we studied whether apical transcytosis of TfR is MT dependent. Indeed, depolymerization of MTs by cold and nocodazole significantly inhibited apical transcytosis of TIR in AP-1B KD MDCK cells (from 16% to 8%, $P < 0.001$) (Figure 1C left) and increased basolateral recycling (from 71% to 77%, $P < 0.05$) (Figure 1C middle). As transcytosis of pIgR also depends on rab11a, we studied whether this was also the case for the transcytosis of endogenous TfR in AP-1B KD MDCK cells. To this end, we generated a tetracycline-repressible MDCK cell line stably expressing a GTP binding-deficient mutant of rab11a (S25N) tagged with monomeric Cherry fluorescent protein (mCh-DN-rab11a). This approach allows efficient overexpression of mCh-DN-rab11a only after the monolayer polarity is established, thus circumventing the disrupting effects of this protein during polarity development (Datta et al., 2011). In the presence of doxycycline, mCh-DN-rab11a expression was much lower than that of endogenous rab11a, whereas removal of the drug induced a 4 x increase of mCh-DN-rab11a expression to a level comparable to the endogenous protein, thus avoiding non-specific effects associated with massive overexpression (Figure 1D). We transiently knocked down μ1B in these cells using electroporation of siRNA. RT-PCR analysis showed that transient knockdown of μ1B-KD was effective, albeit less efficient than in permanently transfected MDCK cells (Figure 1E), resulting in a smaller but statistically significant apical transcytosis of SulfoTag-Tf (Figure 1F). Importantly, expression of mCh-DN-rab11a caused a significant reduction of apical transcytosis of SulfoTag-Tf in transiently AP-1B KD MDCK cells (from 7% to 5%, $P < 0.05$) (Figure 1E), suggesting that rab11a regulates this pathway. Expression of mCh-DN-rab11a did not impair the accuracy of basolateral recycling of SulfoTag-Tf in WT MDCK cells (Supplementary Figure S1D) or the transepithelial electrical resistance (not shown), indicating that mCh-DN-rab11a did not affect establishment of membrane polarity.

These experiments demonstrate that polarized AP-1B KD MDCK cells transport basolateral TIR to the apical PM via a transcytotic route that depends on MTs and rab11a.

**Apical transcytosis of TIR in AP-1B KD MDCK cells involves both ARE and ASE**

To identify the compartments involved in the transcytotic pathway of TIR, we carried out basolateral uptake experiments with fluorescent Tf and studied its colocalization with specific endosomal markers in WT and AP-1B KD MDCK cells. In order to facilitate detection of endogenous TIR, dog Tf was labelled with CF594 (594-Tf), which yielded 3 to >10 times stronger signal than the routinely used Alexa488-Tf in the relevant concentration range ($10^{-14}$ to $10^{-12}$ mol per well), as revealed by spectrophotometry analysis (Supplementary Figure S2A). The specificity of 594-Tf for dog TIR was confirmed by colocalization experiments with anti-TIR antibody (Supplementary Figure S2B) and by competition experiments with unlabelled Tf (not shown).

AP-1B KD MDCK cells incubated basolaterally with 594-Tf for 15 min displayed strong colocalization of 594-Tf with immunostained rab11a at ARE, which typically appeared as a bright spot under the apical PM (Figure 2A, column 3). This colocalization was not observed in AP-1B KD MDCK cells pretreated with nocodazole/cold (Figure 2A, column 4). In contrast, basolaterally internalized 594-Tf was not detected in ARE in WT MDCK cells in the absence or presence of
Anergic endosomes (ARE) are microtubule-dependent, apical-to-basolateral transcytotic vesicles that begin their journey in the apical cytoplasm and proceed to the basolateral membrane. AREs have been shown to transport proteins such as transferrin receptor (TfR) from the apical to the basolateral surface of epithelial cells. The MT motor(s) that mediate this transcytosis across the basolateral membrane are largely unknown.

MT disruption disperses AREs in MDCK cells. In AP-1B KD MDCK cells, 594-Tf was similarly delivered to the apical membrane (Figure 2A, columns 1 and 2). Quantitative analysis using the Mander’s colocalization coefficient showed that the percentage of rab11a colocalizing with 594-Tf increased from 0 ± 0.1% in WT MDCK to 34 ± 3% in AP-1B KD cells (P < 0.001) and was reduced to 0.3 ± 0.2% by nocodazole/cold treatment (P < 0.001) (Figure 2A’ top). Accordingly, the percentage of 594-Tf colocalizing with rab11a increased from 0 ± 0.1% in WT MDCK cells to 7 ± 0.7% (P < 0.001) in AP-1B KD MDCK cells and was reduced to 1.4 ± 0.3% (P < 0.001) after nocodazole/cold treatment (Figure 2A’ bottom). That the subapical rab11a-positive spot was indeed AREs was confirmed by the displacement of this spot to the apicolateral junction under treatment with the MT-stabilizing drug taxol (Supplementary Figure S3A), a typical behaviour of AREs (Casanova et al., 1999; Lapierre et al., 2003). Notably, taxol treatment did not prevent transport of 594-Tf to the laterally displaced ARE in AP-1B KD MDCK cells (Supplementary Figure S3A,A’). Taken together, these results demonstrate that AP-1B KD MDCK cells carry out MT-mediated transcytosis of basolateral TfR to ARE.

Apical sorting endosomes (ASE) appear as multiple small dots located immediately under the apical membrane upon incubation for 5 min with apical 488-WGA (Leung et al., 2000; Cresawn et al., 2007) (Figure 2B). Similar experiments to those performed for ARE, indicated that AP-1B KD MDCK cells transport significantly more basolaterally internalized 594-Tf than WT MDCK cells to ASE (Figure 2B, columns 1 and 3). Transport of basolaterally internalized 594-Tf to ASE was inhibited by nocodazole/cold treatment (Figure 2B, columns 3 and 4). Quantitative analysis showed that the percentage of 488-WGA colocalizing with 594-Tf increased from 4 ± 0.2% in WT MDCK cells to 5 0.7% (P < 0.001) in AP-1B KD MDCK cells and was reduced to 3 ± 0.3% (P < 0.001) after nocodazole/cold treatment (Figure 2B’ top). Accordingly, the percentage of 594-Tf colocalizing with 488-WGA increased from 0.6 ± 0.1% in WT MDCK cells to 7 ± 0.8% (P < 0.001) after nocodazole/cold treatment (Figure 2B’ bottom).
in AP-1B KD MDCK cells and was reduced to 3.6 ± 0.4% (P < 0.001) by nocodazole/cold treatment (Figure 2B' bottom).

Control experiments indicated that basolaterally internalized 594-Tf also reached ASE in the presence of the iron chelator deferoxamine added to the apical medium (Supplementary Figure S3B,B'), ruling out the possibility that 594-Tf was delivered to the apical membrane and subsequently re-internalized into ASE. The amount of 594-Tf transported to ARE and ASE did not increase with longer incubation times (e.g., 30 min) indicating that the transport of basolateral 594-Tf to ARE and ASE reached steady state after ~15 min (Supplementary Figure S3C,C' and D,D').

The experiments reported in this section demonstrate that the transcytotic route of TfR in AP-1B KD MDCK cells involves MT-mediated transport to both classes of apical endosomes, ARE and ASE. In contrast, plgR displayed an extensive colocalization with ARE, as previously reported (Apodaca et al., 1994; Brown et al., 2000), but colocalized poorly with ASE (Supplementary Figure S4D,D'). Our experiments demonstrate that the transcytotic pathways of TfR in AP-1B KD MDCK cells and plgR involve different endosomal itineraries and unravel a novel role for ASE in apical transcytosis.

**LDL receptor undergoes apical transcytosis to ARE in AP-1B KD MDCK**

We next studied whether the apical transcytotic route utilized by TfR in AP-1B KD MDCK cells is also utilized by other AP-1B cargoes. Like TfR, LDLR is basolateral in WT MDCK and non-polar in AP-1B KD MDCK cells and LLC-PK1 cells (Folsch et al., 1999; Gan et al., 2002; Gravotta et al., 2007). In contrast, the bicarbonate transporter NCB1 localizes basolaterally in kidney proximal tubule cells indicating that its basolateral localization mechanism is independent of AP-1B (Li et al., 2007).

For these experiments, we studied the expression of basolateral proteins tagged with GFP. TfR–GFP colocalized poorly with rab11a in WT MDCK cells and extensively in AP-1B KD MDCK cells (Figure 3A), confirming the results obtained with endogenous TfR. LDLR–GFP also extensively increased its colocalization with rab11a in AP-1B KD MDCK cells (Figure 3B). In contrast, NCB1–GFP colocalized poorly with rab11a in both WT and AP-1B KD MDCK cells. Quantitative analysis showed that the percentage of rab11a colocalizing with TfR–GFP increased from 4 ± 1% in WT MDCK to 42 ± 7% in AP-1B KD (P < 0.001). The percentage of rab11a colocalizing with LDLR–GFP also increased from 6 ± 2% to 50 ± 8% (P < 0.001), whereas the percentage of rab11a colocalizing with NCB1–GFP did not increase (6 ± 4% and 5 ± 3%, NS). Accordingly, the percentage of TfR–GFP colocalizing with rab11a increased from 2 ± 0.8% to 13 ± 2% (P < 0.001). The percentage of LDLR–GFP colocalizing with rab11a also increased from 5 ± 1% to 24 ± 5% (P < 0.001), whereas the percentage of NCB1–GFP colocalizing with rab11a did not increase (4 ± 3% and 3 ± 1%, NS) (Figure 3A'–C').

These results demonstrate that AP-1B cargoes undergo transcytosis via ARE in AP-1B KD MDCK cells, whereas AP-1B independent cargos remain strictly basolateral and avoid ARE in AP-1B KD MDCK cells.

**Transport of basolateral TfR to ARE requires the plus-end microtubule motor KIF16B**

To date, the MT motor(s) that mediate transcytosis across epithelia remain unknown. We used a candidate approach to search for MT motors involved in the transcytosis of TfR.
KIF16B mediates transcytosis of the apical protein p75-neurotrophin receptor (p75) in MDCK cells (Jaulin et al., 2007). KIF16B was discovered in a search for kinesins that bind the endosomal lipid PI3P and found to mediate TIR recycling in fibroblastic cells (Hoepfner et al., 2005). Transfection of a truncated, motor-less version of KIF16B (DN-KIF5B-CFP) that blocks biosynthetic trafficking of p75 did not prevent transport of basolaterally internalized 594-Tf to ARE in AP-1B KD MDCK cells (Figure 4B). In contrast, transfection of a motor-less form of KIF16B (DN-KIF16B-YFP) that inhibits TIR recycling in HeLa cells (Hoepfner et al., 2005) prevented transport of basolaterally internalized 594-Tf to ARE in AP-1B KD MDCK cells (Figure 4A). Quantitative analysis showed that the percentage of rab11a colocalizing with 594-Tf increased from 0.3 ± 0.2% in untransfected WT MDCK cells to 32 ± 3% (P < 0.001) in untransfected AP-1B KD MDCK cells and was reduced to 12 ± 3% (P < 0.001) by the expression of DN-KIF16B-YFP, but not by the expression of DN-KIF5B-CFP (Figure 4C, left). Accordingly, the percentage of 594-Tf colocalizing with rab11a increased from 0 ± 0.1% in untransfected WT MDCK cells to 8 ± 1% (P < 0.001) in untransfected AP-1B KD MDCK cells and was reduced to 2 ± 1% (P < 0.05) by expression of DN-KIF16B-YFP, but not by DN-KIF5B-CFP in AP-1B KD MDCK cells (Figure 4C, right).

Because antibodies against canine KIF16B are not available, we investigated the expression of KIF16B in MDCK cells by RT-PCR analysis using two different sets of primers. These experiments showed that KIF16B is endogenously expressed by both WT and AP-1B KD MDCK cells; furthermore, the sequence of the amplified product blasted exclusively with the canine KIF16B mRNA (Supplementary Figure S5).

We next investigated whether knockdown of endogenous KIF16B in MDCK cells inhibits the apical transcytosis of basolaterally internalized TIR. Treatment of both WT and AP-1B KD MDCK cells with a pool of four siRNAs against KIF16B decreased its expression by 78% (Figure 4D’ and Supplementary Figure S5C). KD of KIF16B in AP-1B KD MDCK cells caused a significant reduction in the localization of 594-Tf with immunostained rab11a at ARE after incubation with basolateral 594-Tf for 15 min (Figure 4D, columns 3 and 4), although this reduction was less pronounced than that observed after treatment with DN-KIF16B-YFP. In contrast, 594-Tf was virtually not detected in ARE in WT MDCK cells transfected with luciferase or KIF16B siRNA (Figure 4D, columns 1 and 2). Quantitation of these experiments showed that the percentage of rab11a colocalizing with 594-Tf increased from 3 ± 0.4% in WT-Luc KD to 29 ± 2% in AP-1B KD-Luc KD (P < 0.001) and was reduced to 11 ± 1% in AP-1B KD-KIF16B KD MDCK cells (P < 0.001) (Figure 4D’ left). Accordingly, the percentage of 594-Tf colocalizing with rab11a increased from 0.4 ± 0.1% in WT-Luc KD to 6 ± 0.6% in AP-1B KD-Luc KD (P < 0.001) and was reduced to 4 ± 0.4% in AP-1B KD-KIF16B KD MDCK cells (P < 0.05) (Figure 4D’ right).

These results show that KIF16B mediates transcytosis of TIR to ARE in AP-1B KD MDCK cells.

**KIF16B mediates trafficking of TIR from CRE to ARE using non-centrosomal microtubules**

From which compartment does KIF16B mediate apical transcytosis of TIR to ARE? Experiments in non-polarized cells have shown that KIF16B regulates TIR recycling at the level of SE (Hoepfner et al., 2005); however, apical transcytosis of pIgR occurs from CRE to ARE (Apodaca et al., 1994; Brown et al., 2000). Hence, we designed experiments to determine which candidate endosomal compartments contain KIF16B.

As CRE has no specific marker, we identified this compartment from BSE using a 2-colour pulse-chase protocol using 594-Tf (red) and 633-Tf (green) (Supplementary Figure S6). MDCK cells were exposed basolaterally to 633-Tf for 25 min and to 594-Tf for 5 additional minutes. Under these conditions, 633-Tf labelled preferentially CRE and 594-Tf labelled preferentially BSE. Using this protocol, we observed that WT-KIF16B colocalized to a significantly higher extent with CRE than with BSE (Figure 5A arrows and quantification analysis in Figure 5A’), suggesting that KIF16B operates at CRE. Importantly, DN-KIF16B did not inhibit the segregation of 633-Tf and 594-Tf during the 5-min chase (Figure 5B), indicating that KIF16B is not involved in traffic of TIR from BSE to CRE. DN-KIF16B, which lacks the motor domain and therefore can attach to the proximal compartment (through the C-terminus region) but cannot traffic to the distal compartment, colocalized preferentially with CRE (Figure 5B arrows and Figure 5B’), suggesting that CRE is the proximal compartment of this motor. Furthermore, DN-KIF16B did not alter the morphology of BSE and CRE. Similar results were obtained in experiments carried out in AP-1B KD MDCK cells (Figures 5C, 5D and 5E).

Given that KIF16B is a plus-end kinesin and that the centrosome and apical endosomes localize more apically than CRE in polarized MDCK cells, transport of basolateral TIR to CRE requires reorientation of apical endosomes. Therefore, we designed experiments to determine whether the Golgi apparatus acts as a MT-organizing centre (Chabin-Brion et al., 2001) and is in intimate spatial relationship with CRE in polarized MDCK cells (our own unpublished observations) and with RE in non-polarized cells (REs have been denominated the para-Golgi compartment) (Yamashiro et al., 1984). We therefore carried out MT regrowth assays (by adding and removing nocodazole) in polarized WT MDCK cells (see Materials and methods) to determine whether the Golgi apparatus acts as a MT-organizing centre in polarized MDCK cells. These experiments showed MT growing from Golgi nucleation sites (Figure 5E, arrowhead) more basally localized in the centrosome (Figure 5E, arrow). As these non-centrosomal MTs grow with their plus ends oriented mainly towards the subapical cytoplasm, they are likely to be involved in KIF16B-mediated transport of TIR from juxta-Golgi CRE to subapical ARE.

Taken together, these experiments support a model in which AP-1B KD MDCK cells transcytose basolateral TIR from CRE to ARE utilizing the plus-end kinesin KIF16B, likely using non-centrosomal MTs emerging from the Golgi complex (see model in Figure 8A). As parallel experiments showed that KIF16B does not mediate transcytosis of pIgR (Supplementary Figures S4A,B,C), our results indicate that parallel transcytotic routes exist in MDCK cells, with remarkable selectivity for different cargoes.

**AP-1B-deficient RPE displays apical transcytosis of TIR**

The RPE constitutes the main barrier between the blood and the retina and hence performs many functions essential for the survival of the retinal photoreceptors (Strauss, 2005). Indeed, RPE cells take up iron from the neural retina through their apical membrane, thus preventing the accumulation of toxic iron.
KIF16B-mediated transferrin receptor transcytosis

AE Perez Bay et al

Figure 4 Transport of basolateral TfR to ARE requires the plus-end microtubule motor KIF16B. (A, B) WT and AP-1B KD MDCK cells transiently transfected with DN-KIF16B-YFP (a) or DN-KIF5B-CFP (b) were incubated from the basolateral surface with fluorescent 594-Tf for 15 min and immunostained with anti-rab11a. Transfected and non-transfected cells can be identified in the bottom panel by the signal of DN-KIF16B-YFP or DN-KIF5B-CFP. (C) Cells from experiments represented in (a) and (b) were quantified for the percentage of pixels of rab11a colocalizing with 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with rab11a (right). (D) WT and AP-1B KD MDCK cells nucleofected with either luciferase or KIF16B siRNA were incubated from the basolateral surface with fluorescent 594-Tf for 15 min and immunostained with anti-rab11a. (D') Cells from experiments represented in (d) were quantified for the percentage of pixels of rab11a colocalizing with 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with rab11a (right). (D'') RNA levels of KIF16B and GAPDH in AP-1B KD cells nucleofected with luciferase or KIF16B siRNA. NS, no significance, *P<0.05, **P<0.001. Red line represents the median. Scale bar, 10 μm.
levels of this metal (Yefimova et al., 2000; He et al., 2007; Garcia-Castineiras, 2010). As we have recently shown that RPE constitutively lacks μ1B (Diaz et al., 2009), we investigated whether RPE cells transcytoses Tf to the apical PM through the apical transcytotic route we demonstrated in AP-1B KD MDCK cells. For these

Figure 5 KIF16B mediates trafficking of Tf from CRE to ARE. WT MDCK cells transiently transfected with the WT-KIF16B-YFP (full-length KIF16B) (A) or the DN-KIF16B-YFP (motorless KIF16B) (B) were incubated for 25 min from the basolateral surface with 633-Tf (green) followed by a 5 min chase, in which 594-Tf (red) was added to the basolateral medium. The left panel displays the signal of 594-Tf and 633-Tf from the same field. Arrows emphasize endosomes containing KIF16B and 633-Tf, but not 594-Tf. (A', B') Cells from experiments represented in A and B were quantified for the percentage of pixels of the kinesin colocalizing with 633-Tf or 594-Tf (top) and the percentage of pixels of 633-Tf or 594-Tf colocalizing with the kinesin (bottom) (C, C', D, D'). The same experiment described in A, A', B and B' was carried out in AP-1B KD MDCK cells. (E) WT MDCK cells were treated with nocodazole/cold at 4 °C, incubated at 37 °C in the absence of the drug for MTs regrowth (see Methods), fixed and immunostained with markers of MTs (α-tubulin), golgi (giantin) and centrioles (γ-tubulin). *P < 0.05, **P < 0.001. Red line represents the median. Scale bar, 10 μm.
Figure 6  AP-1B-deficient retinal pigment epithelium displays apical transcytosis of TfR. (A) Values of apical transcytosis, basolateral recycling and the intracellular pool of SulfoTag-Tf after 60 min of recycling in WT MDCK and ARPE-19 cells. (B) WT MDCK or ARPE-19 cells were incubated from the basolateral side with fluorescent 594-Tf for 30 min and immunostained with anti-rab11a. (B') Cells from experiments represented in (b) were quantified for the percentage of pixels of rab11a colocalizing with 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with rab11a (right). (C) WT MDCK or ARPE-19 cells were incubated with fluorescent 594-Tf as in (b) and stained for ASE with incubation of 488-WGA from the apical surface for 5 min at 37°C and washed with NADG at 4°C. (C') Cells from experiments represented in C were quantified for the percentage of pixels of 488-WGA colocalizing with 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with 488-WGA (right). (D) ARPE-19 fully polarized in Transwell filters were electroporated with DN-KIF16B-YFP (Deora et al., 2007), incubated from the basolateral surface with fluorescent 594-Tf for 15 min and immunostained with anti-rab11a. Transfected and non-transfected cells can be identified in the bottom panel by the signal of DN-KIF16B-YFP. (D') Cells from experiments represented in (d) were quantified for the percentage of pixels of rab11a colocalizing with the 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with rab11a (right). Bars represent mean ± standard error.*P<0.05, **P<0.001. Red line represents the median. Scale bar, 10 μm.
Figure 7 Expression of AP-1B in fibroblastic CHO cells generates a rab11a-independent recycling route. (A) WT and AP-1B KD subconfluent MDCK were incubated with fluorescent (dog) 594-Tf for 50 min and immunostained for rab11a. (A’) Quantification of the percentage of pixels of rab11a colocalizing with 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with rab11a (right). (B) WT and AP-1B (+) CHO cells were incubated with fluorescent (human) 594-Tf for 50 min and immunostained with anti-rab11a and anti-HA (µB-HA). (B’) Quantification of the pixels of rab11a occupied by 594-Tf (left) and the pixels of 594-Tf occupied by rab11a (right). (C) Western blot analysis showing expression of stably transfected µB-HA and/or transiently transfected mCh-DN-rab11a in CHO cells. (C) TfR recycling assay: after 60 min uptake of SulfoTag-Tf, cell were either allowed to recycle for 56 min or lysed immediately afterwards (0-min recycling) (see Methods). Retention was calculated as ‘retention after 56-min recycling/retention after 0-min recycling’, and normalized to ‘control WT CHO’. Bars represent mean ± standard error. NS, no significance, *P<0.05, **P<0.001. Red line represents the median. Scale bar, 10 µm.

In experiments, we utilized monolayers of the human RPE cell line ARPE-19, which develop tight junctions and a polarized distribution of the monocarboxylate transporters MCT1 and MCT4 (Supplementary Figures S7A,B) similar to that observed in RPE in vivo. Biochemical SulfoTag-Tf recycling assays (50 min) showed that, compared to WT MDCK cells, RPE cells displayed significantly higher apical transcytosis (15% versus 3%, P<0.001), lower basolateral recycling (32% versus 76%, P<0.001) and higher intracellular retention (52% versus 22%, P<0.05) (Figure 6A). Microscopic uptake assays using human 594-Tf showed that after 30 min of basolateral internalization, RPE cells display a large amount of 594-Tf in ARE (which in these cells appear as a bright spot under the apical surface), where it colocalizes with rab11a (Figure 6B). Colocalization of 594-Tf with rab11a was significantly higher than that observed in control WT MDCK cells (Figure 6B’). Similarly, after 30 min, RPE cells displayed extensive colocalization of 594-Tf with apically internalized 488-WGA (Figure 6C), a marker of ASE, significantly higher than that of WT MDCK cells (Figure 6C’). Transfection of DNI-KIF16B-YFP (Hoepfner et al., 2005) prevented transport of basolaterally internalized 594-Tf (15 min) to ARE in ARPE-19 cells (Figure 6D), as previously shown for AP-1B KD MDCK cells. Quantification of these experiments showed that the percentage of rab11a colocalizing with 594-Tf decreased from 34±4% to 10±2% (P<0.001) by the expression of DNI-KIF16B-YFP in ARPE-19 cells. Accordingly, the percentage of 594-Tf colocalizing with rab11a decreased from 8±1% to 1±0.1% (P<0.001) by expression of this mutant (Figure 6D’).

These results show that constitutively µB-deficient RPE cells transcytose a substantial fraction of basolateral TIR to ARE in a KIF16B-dependent manner, as we have shown in AP-1B KD MDCK cells. This route is likely responsible for the presence of high levels of TIR in the apical PM of RPE, necessary for iron homeostasis in the retina.

Expression of AP-1B in CHO cells generates a rab11a-independent recycling route

The experiments described above demonstrate that lack of expression of AP-1B promotes incorporation of TIR into a rab11a-dependent transcytotic route to the apical surface in MDCK and RPE cells and suggest that AP-1B plays a major role in the constitutive recycling of TIR to the basolateral membrane.
role in generating a rab11a-independent recycling route for TfR in polarized epithelia. Is this also true for non-polarized MDCK cells? Experiments following the internalization of 594-Tf for 30 min in subconfluent MDCK cells (Figure 7A) showed that indeed, expression of AP-1B prevents access of TfR to rab11a-positive endosomes, which are localized to the perinuclear region of these cells. Quantitative analysis indicated that the percentage of rab11a colocalizing with 594-Tf increased from 6 ± 1% in non-polarized WT MDCK cells to 42 ± 3% (P < 0.001) in non-polarized AP-1B KD MDCK cells (Figure 7A’, left). Accordingly, the percentage of 594-Tf colocalizing with rab11a increased from 5 ± 1% in non-polarized WT MDCK cells to 15 ± 3% (P < 0.05) in non-polarized AP-1B KD MDCK cells (Figure 7A’, right). These results clearly show that the presence of AP-1B is sufficient to restrict recycling TfR from reaching rab11a-positive endosomes even before polarity develops and suggest that subconfluent MDCK cells recycle different cargoes through AP-1B and rab11a recycling endosomes.

We speculated that expression of AP-1B might also be sufficient to generate an alternative recycling route for TfR in fibroblastic CHO cells, which normally recycle TfR along a rab11a-dependent general recycling pathway. To test this hypothesis, we expressed the epithelial-specific subunit μ1B tagged with a HA epitope (μ1B-HA) in a CHO cell line in which hamster TfR had been replaced for human TfR (McGraw et al., 1987). As CHO cells express the ubiquitous adaptor AP-1A, which shares three of its four subunits (β1, σ1 and γ1), these cells were able to assemble AP-1B as demonstrated by the almost complete colocalization of μ1B-HA with endogenous γ1 subunit (Supplementary Figure S8A, A’). In addition, WT and AP-1B (+) CHO cells expressed similar levels of rab11a (Supplementary Figure S9).

Expression of AP-1B in fibroblastic CHO cells did not reduce the colocalization of internalized 594-Tf with rab11a (Figure 7B arrows, Figure 7B’), indicating that AP-1B is not sufficient to divert these proteins in CHO cells. However, μ1B-HA colocalized extensively at perinuclear RE with internalized 594-Tf (Supplementary Figure 8B, B’), suggesting that AP-1B might be functionally involved in TfR recycling. To test this hypothesis, mCh-DN-rab11a was transiently transfected in WT and AP-1B (+) CHO cells (Figure 7C) and recycling assays were carried out. As previously reported (Ullrich et al., 1996; Ren et al., 1998), mCh-DN-rab11a inhibited TfR recycling by 60% in WT CHO cells (n = 6, P < 0.001). However, mCh-DN-rab11a did not inhibit TfR recycling in AP-1B (+) CHO cells (Figure 7C’).

These results suggest that expression of AP-1B in CHO cells is sufficient to generate a rab11a-independent TfR recycling pathway but not sufficient to generate geographically segregated TfR and rab11a endosomes (see model in Figure 8B). They also suggest that the AP-1B-dependent pathway in CHO cells is equivalent to the basolateral pathway of epithelial cells, whereas the rab11a pathway is equivalent to the apical-recycling pathway of epithelial cells.

**Discussion**

It is well known that epithelial cell lines constitutively lacking AP-1B or silenced of the epithelial-specific subunit μ1B

---

**Figure 8** Model. (A) WT MDCK cells recycle most TfR to the basolateral membrane through BSE and CRE. In contrast, in AP-1B KD MDCK cells a substantial fraction of TfR is trafficked from CRE to ARE by KIF16B and non-centrosomal MTs and delivered to the apical PM in a rab11a-mediated manner. (B) WT CHO cells recycle TfR through perinuclear RE in a rab11a-mediated manner. In contrast, CHO cells stably transfected with AP-1B generate a rab11a-independent route, likely mediated by AP-1B.
express basolateral proteins at the apical PM (Folsch et al., 1999; Gan et al., 2002; Gravotta et al., 2007); however, the route and mechanisms responsible for the apical ‘missorting’ of basolateral proteins are still unknown. Elucidating the nature of this route and mechanisms has become more urgent with the discovery of two native epithelia, the RPE and the kidney proximal tubule, that lack AP-1B and depend on apically expressed basolateral proteins to perform their normal physiological functions (Diaz et al., 2009; Schreiner et al., 2010). Using the sensitive biochemical and microscopic techniques that we developed here to study the recycling of endogenous dog TIR, we show that AP-1B KD MDCK cells divert TIR from its basolateral recycling circuit into an apical transcytotic pathway with distinctive features that differentiate it from the well-studied transcytotic pathway of plgR. The transcytotic route of TIR, like that of plgR, traverses ARE and depends on rab11a. Surprisingly (and in contrast to plgR), TIR traverses ASE, an apical endocytic compartment (Bomsel et al., 1989) previously shown to participate in the biosynthetic route of raft-associated PM proteins (Cresawn et al., 2007; Weiss and Rodriguez-Boulan, 2009). The ‘missorting pathway’ is also utilized by other AP-1B-dependent basolateral cargoes (e.g., low-density lipoprotein receptor, LDLR), but not by AP-1B independent basolateral cargoes (NBC1) (Figure 3).

We found that MT integrity is necessary for transcytosis of TIR to ARE, as previously shown for plgR (Apodaca et al., 1994; Brown et al., 2000). Strikingly, we also found that the plus-end kinesin KIF16B transports TIR from CRE to ARE (where the bulk of it resides) to ARE, likely utilizing MT nucleated in the Golgi, which is in intimate relationship with the CRE. As this motor does not mediate transcytosis of plgR to ARE, our results indicate that different apical transcytotic pathways exist in epithelial cells, with remarkable selectivity for different cargoes (Figures 4, 5 and 8).

KIF16B was previously shown by the Zerial group to be capable of binding, through a Px domain, the early endosomal protein rab11, and to regulate the recycling of TIR in fibroblastic cells (Hoepfner et al., 2005). More recently, Hirokawa and coworkers showed that KIF16B binds to and traffics FGF receptor vesicles to the PM and that rab14 might be necessary for both processes (Ueno et al., 2011). As Wilson and coworkers have shown that rab14 regulates apical trafficking of the raft-associated protein MAL (Cheong et al., 1999; Puertollano et al, 1999; Kitt et al., 2008), future work is necessary to determine whether rab14 participates in the transcytotic route of TIR in AP-1B KD MDCK cells. Rab4 might also be involved in the pathway reported here, as it has been previously shown to increase apical transcytosis of TIR when overexpressed in WT MDCK cells (Mohrmann et al., 2002). However, the mechanism involved is unclear given that rab4 is believed to function at BSE, which are proximal to the site of action of AP-1B.

Recent observations from our laboratory suggest that AP-1B mediates basolateral recycling of TIR from CRE to the basolateral membrane through the binding of its medium subunit μ1B to a sorting signal in a 40-amino-acid stretch in the cytoplasmic domain of TIR (Odorizzi and Trowbridge, 1997; Gravotta et al., 2012). Because loss of AP-1B results in trafficking of TIR to the apical membrane rather than retention at CRE, TIR might express recessive apical signals. Previous work has shown that apical transcytosis of the plgR in WT MDCK cells is mediated by N-glycans acting as apical signals (Luton et al., 2009). TIR contains three N-glycosylation and one O-glycosylation sites and our preliminary results (Perez Bay, Schreiner and Rodriguez-Boulan) suggest that N-glycans are involved in guiding its apical transcytosis in AP-1B-deficient epithelia.

The apical transcytotic pathway for cognate basolateral proteins we have identified in AP-1B KD MDCK cells, is likely to play an important role in the organization and function of native epithelia that lack AP-1B, for example, RPE and kidney proximal tubule (Diaz et al., 2009; Schreiner et al., 2010), due to the apical expression of basolateral proteins that perform important physiological functions for the host organs. Indeed, this pathway is present in RPE cells (ARPE-19 cells), which constitutively lack AP-1B and display a similar, although more pronounced, phenotype as AP-1B KD MDCK cells. Notorious features of this phenotype were the reduction of basolateral recycling and the increased intracellular pool of TIR, which support the role of AP-1B in promoting recycling of this receptor back to the (basolateral) PM. The stronger phenotype of ARPE-19, compared to AP-1B KD MDCK cells, might be due to incomplete KD of AP-1B in MDCK cells or a compensatory mechanism that occurs in AP-1B KD cells, but not in cells that constitutively lack this adapter.

A long standing question in the epithelial trafficking field is: which pathway (apical or basolateral) is mechanistically equivalent to the general trafficking route of fibroblastic cells? The basolateral biosynthetic route was initially hypothesized to fulfill this role because many receptors that mediate exchanges with the blood on the PM of fibroblastic cells localize to the basolateral PM of epithelial cells (Simons and Fuller, 1985; Fuller and Simons, 1986). However, the discovery that rab11a, a major regulator of the general recycling route of non-polarized cells, localizes to subapical ARE, which, like the RE of fibroblastic cells, is clustered by MT motors around the centrosome, suggested the opposite hypothesis, that is, that the apical route of epithelial cells is equivalent to the general trafficking route of fibroblastic cells (Zacchi et al., 1998; Wang et al., 2000). However, a critical piece of functional evidence in support of this hypothesis has been missing up to date, that is, that a protein recycled by a rab11a-dependent mechanism in fibroblastic cells can traffic along the rab11a-dependent apical route in epithelial cells. Our results, based on the structural and functional analysis of TIR recycling pathways in AP-1B (+) and AP-1B (−) epithelia, support this hypothesis as they demonstrate that apically recycling TIR in AP-1B KD MDCK cells traverses rab11a-positive recycling endosomes and depends on rab11a for surface delivery, exactly as TIR utilizing the general recycling pathway of fibroblastic cells (Ullrich et al., 1996; Ren et al., 1998).

Materials and methods

Reagents

The following antibodies were used in this study: rabbit anti-rab11a (Invitrogen, Carlsbad, CA), mouse anti-TIR (Zymed, San Francisco, CA), mouse anti-β1 integrin (Roche, Indianapolis, IN), rat anti-γ adaptin (BD Biosciences, San Jose, CA), rat anti-β tubulin (kindly provided by Dr Geri Kreitzer), rabbit anti-γ tubulin (Sigma-Aldrich, Saint Louis, MO), mouse anti-Giantin (kindly provided by Dr Hauri), sheep anti-secretory component (kindly provided by Dr Mostov). 488-WGA was purchased from Sigma-Aldrich and 647-WGA from Invitrogen. Nocodazole was from Sigma-Aldrich.
Plasmids
Generation of monomeric Cherry-tagged dominant-negative rab11a (mCh-DN-Rab11a): first, we obtained the tetracycline-responsive promoter from the pTR-mega vector (Clontech, Mountain View, CA) with NruI and EcoRV. Second, we removed the CMV promoter from the pcDNA3.1 hygro (+) vector ( Invitrogen ) and inserted the tetracycline-responsive promoter with Xho I. Third, we inserted the Ch-DN-Rab11a (kindly provided by Dr Ching-Hwa Sung) into the above vector using KpnI and XbaI sites. The NBC1–GFP plasmid was generated by cloning kidney NBC1 (kindly provided by Manoocher Soleimani) with Nhel and KpnI into pEGFP-N1 (Clontech).
The DN-KIF16B-YFP and DN-KIF5B-CFP plasmids were kindly provided by Marino Zerial and Geri Kreitzer.

Cell culture
MDCK cells were maintained in DMEM (Thermo Scientific, Pittsburgh, PA) supplemented with 5% FBS (Invitrogen). Try-v1 cells (CHO strain expressing the human TIR, kindly provided by Dr Tim McGraw) were maintained in 10% FBS DMEM. Human ARPE-19 cells (ATCC, Bethesda, MD) were maintained in RPMI media: MEMα (Sigma-Aldrich) supplemented with 1% FBS, N1 supplement (100 × , Sigma-Aldrich), glutamine–penicillin–streptomycin (100 × , Sigma-Aldrich), nonessential amino-acids (100 × , Sigma-Aldrich), hydrocortisone (20 μg/mL), taurine (250 mg/L), and transferrin–bovine serum albumin (0.15 μg/mL) (THT). Polarized MDCK and ARPE-19 cells were plated at 3 × 10⁴ cells/cm² on 12 mm Transwell chambers and used 4 days or 4–6 weeks after plating, respectively. Non-polarized CHO cells were plated at 1 × 10⁶ cells/cm² on 12-mm well plates and used after 2–3 days; whereas subconfluent MDCK cells were plated at 3 × 10⁴ cells/cm² on 12-mm plate and used after 1 day.

Transgenic cell lines
MDCK cells stably knocked down for μ1B (Gravotta et al, 2007) were grown in the presence of 2.5 μg/ml of puromycin (Sigma-Aldrich). The mCherry-dominant-negative rab11a mCh-DN-Rab11a MDCK cell line was generated by transfection of a T23 MDCK strain expressing the tetracycline repressor ( kindly provided by Dr. Keith Mostov) and maintained with 1 mg/ml hygromycin B (Invitrogen) 1 mg/ml G418 (Medateach, Manassas, VA) and 2.5 μg/ml puromycin. The AP-1B (+) CHO cell line was generated by transfection of Try-v1 cells with a plasmid expressing human μ1B tagged with influenza HA (gift from Dr Heike Folsch and Dr Ir a Mellman) and maintained with 1 mg/ml hygromycin for μ1B expression and G418 for human TIR expression.

siRNA transfection and RT-PCR
To knockdown the μ1B transcript, we used previously published protocol and siRNAs (Gravotta et al, 2007). Briefly, WT MDCK cells in suspension culture were treated with 5 μl of 40 μM siRNA and subjected to three rounds of electroporation with Amaxa Nucleofector kit V, spaced every 3 days, and plated after the last round on Transwell chambers. The extent of μ1B silencing in cells transfected with siRNA or in the stable μ1B-KD cell line was assessed by RT-PCR. A one-step RT-PCR (Qiagen, Valencia, CA) was run under standard conditions for a total of 30 cycles with 200 ng of mRNA per 50-μl reaction. To knockdown the canine KIF16B, we used the following siRNAs (designed with an algorithm from Dharmacon (Waltham, MA, USAJ): 5′-GGTGAAGGATAAA TCGAAATTT-3′, 5′-AGGCAGGATTTGGGAGATT-3′, 5′-GGGCAAG GACGGACGCTT-3′, 5′-AGGAAAGATGGAAGGAAAT-3′: siRNAs were nucleofected using the protocol described above. KIF16B expression was analysed by RT-PCR with the following primers: canine primer set 1: 5′-AAATTTGACGTTGTTGACACCG-3′ and 5′-GCTTTTACGACGACGGCTC-3′, canine primer set 2: 5′-GACCTGCG CTGAGATACAA-3′ and 5′-TCATGGGCCTTACCCTTCAACAG-3′; human primer set 1: 5′-AGTCTGGGATTATGACACAAAAG-3′ and 5′-CCTTGAGCA AGACGGCCTTTT-3′; human primer set 2: 5′-TGACCTTCCGTGTTGTC TCAGA-3′ and 5′-ACCTCAGTGGCCACATTGCTG-3′ (see Supplementary Figure 5a).

Protein labelling
Fluorescent Tf. Dog or human Tf (Sigma-Aldrich) were conjugated with the fluorophores CF594 (Biotium, Hayward, CA) or SeTaula67, SeTaula68 ( SETA Biomedicals, Urbana, IL ) or HiLyte685 (AnaSpec, Fremont, CA) using NHS chemistry. Different dye/protein molar ratios were tested following manufacturers’ indications. Subsequently, fluorescent Tfs were purified with PD10 columns (GE Healthcare, Piscataway, NJ). The samples were purified again with PD10 columns and with 50 KD cut-off filters (Millipore). The brightest reagent (TF labelled with CF594 [594-Tf]) was identified by performing reagent (TF labelled with CF594 [594-Tf]) was identified with a fluorometer. 594-Tf was validated as a ligand for TIR through fluorescence microscopy experiments showing its colocalization with anti-TIR (Supplementary Figure S1B) and through competition experiments that showed inhibition of 594-Tf uptake by the presence of 200 x unlabelled Tf.

SulfoTag-Tf. Dog or human Tf were sequentially conjugated with the luminophore SulfoTag (Mesoscale Discovery, Gaithersburg, MD) and biotin (Sigma-Aldrich), using NHS chemistry and purified with PD10 columns after each reaction. Then, SulfoTag-Tfs were iron loaded, purified again with PD10 columns and with 50 Kd cut-off filters. Different molar ratios (luminophore/protein and/or biotin/protein) were tested following manufacturers’ indications. The best-performing reagent was identified by highest signal/noise ratio in surface binding assays of subconfluent MDCK cells. Non-specific binding was blocked with 200 x unlabelled Tf.

Uptake assays and immunofluorescence
For 594-Tf uptake assays, cells were starved for 60 min at 37 °C in HBSS containing 20 mM HEPES (standard buffer) and incubated for 60 min at 4 °C with 10 μg/ml 594-Tf in 1% BSA standard buffer. Then, temperature was shifted to 37 °C and, after the indicated time, cells were immediately rinsed (with ice-cold HBSS) and fixed ( ice-cold 4% PFA in PBS). After quenching PFA (50 mM NaN3 in PBS) for 15 min, cells were permeabilized with 0.01% triton X-100 in PBS for 7 min, incubated with the corresponding primary antibodies for 45 min and secondary antibodies for 30 min in 1% BSA PBS, and washed three times after each incubation.

To label ASE, cells were exposed from the apical side to fluorescent WGA for 5 min at 37 °C in 1% BSA standard buffer. Residual fluorescent WGA was stripped from the PM by washing three times with 100 mM N-acetyl-D-glucosamine in HBSS for 10 min at 4 °C and then cells were fixed.

When utilized, nucodazole (33 μM), taxol (5 μM) or apical defer-oxamine was applied during starvation until fixation. Tunicamycin (2 μM) was applied 2 h before and during transfection with TfR–GFP (~20h). For MT re-growth assays, cells were chilled at 4 °C for 60 min and treated with nucodazole at 4 °C for additional 120 min. Then cells were permeabilized with 0.5% Triton X-100 in HBSS at 37 °C for 3 min, incubated in HBSS at 37 °C for additional 2 min, fixed with methanol at −20 °C for 30 s and processed for immunofluorescence.

Microscopy
Images were collected with a Zeiss Axio Observer inverted microscope equipped with a Yokogawa Confocal Scanner Unit CSU-X1 and a Photometrics Cool Snap HQ2 Camera using a Zeiss plan-apochromat 40X/1.4 oil-immersion objective. Acquisition and analysis were performed with Axiovision Rel. 4.8 (Zeiss, Oberkochen, Germany) software.

Colocalization analysis and statistics
Pearson’s and Manders’ coefficients are the two most accepted tools to measure colocalization between different cellular markers A and B. Advantages of the Pearson’s coefficient are its simplicity and its independence of signal levels and signal offset. However, it requires the use of two markers to co-occur in a fixed proportion to one another. This is usually not the case for a cargo reaching a compartment, such as TIR, which localizes in various endosomal compartments at...
various levels, not necessarily colocalizing with endosomal markers in a fixed proportion. On the other hand, the Manders' colocalization coefficient does not have this requirement and, importantly, it provides information on two useful components: the fraction of marker 'A' occupied by marker 'B' and the fraction of marker 'B' occupied by marker 'A'. To determine the area of marker 'A' (i.e., rab11a) occupied by marker 'B' ('i.e., S94-Tf), we quantified the pixels of marker 'A' and the fraction of pixels of marker 'A' colocalizing with marker 'B'. This operation was applied for all the confocal sections of a region of interest (i.e., one cell). Then, the area of marker 'A' occupied by marker 'B' was calculated by the formula 'pixels of marker 'A' colocalizing with marker 'B' pixels of marker 'A'. The corresponding formula was utilized to calculate the area of marker 'B' occupied by marker 'A'. We used this method to assess colocalization of the following marker pairs: TfR and rab11a; TfR and WGA; area of marker 'B' occupied by marker 'A'. This operation was applied for all the confocal sections of a region of interest (i.e., one cell). Then, the area of marker 'A' occupied by marker 'B' was calculated by the formula 'pixels of marker 'A' colocalizing with marker 'B' pixels of marker 'A'. The corresponding formula was utilized to calculate the area of marker 'B' occupied by marker 'A'.

We used this method to assess colocalization of the following marker pairs: TfR and rab11a; TfR and WGA; area of marker 'B' occupied by marker 'A'. This operation was applied for all the confocal sections of a region of interest (i.e., one cell). Then, the area of marker 'A' occupied by marker 'B' was calculated by the formula 'pixels of marker 'A' colocalizing with marker 'B' pixels of marker 'A'. The corresponding formula was utilized to calculate the area of marker 'B' occupied by marker 'A'. We used this method to assess colocalization of the following marker pairs: TfR and rab11a; TfR and WGA; area of marker 'B' occupied by marker 'A'. This operation was applied for all the confocal sections of a region of interest (i.e., one cell). Then, the area of marker 'A' occupied by marker 'B' was calculated by the formula 'pixels of marker 'A' colocalizing with marker 'B' pixels of marker 'A'. The corresponding formula was utilized to calculate the area of marker 'B' occupied by marker 'A'.

Statistics

Values were expressed as mean ± standard error. The statistical method used was the Student's t-test. The levels of statistical significance are specified for each comparison in the respective figure.

Biochemical assays

Recycling/transcytosis. Polarized MDCK or ARPE-19 cells were starved with standard buffer for 60 min at 37°C and incubated with 10 μg/ml SulfoTag-Tf (dog and human, respectively) in 1% BSA standard buffer for 60 min at 37°C from the basolateral side only. SulfoTag-Tf was removed from the PM at 4°C with: (i) three rinses (1% BSA standard buffer), (ii) 30 min incubation with pH 5 buffer (200 mM NaCl, 50 mM 2-(N-morpholino)ethanesulfonic acid, pH 5) plus 50 μM deferoxamine (EMD Bioscience, Rockland, MA), (iii) 30 min incubation with 200X unlabelled Tf in 1% BSA standard buffer and (iv) three additional rinses with 1% BSA standard buffer. Cells were incubated for the indicated times at 37°C with recycling buffer (200 μl unlabelled Tf in 1% BSA standard buffer). Samples were collected from the basolateral and apical chambers and from cell lysates, and quantified in a SI2400 Multiplex analyser (Meso Scale Discovery). Non-specific binding was determined by incubating the cells with SulfoTag-Tf in the presence of 200 μl excess unlabelled human Tf. When specified, nocodazole (Sigma-Aldrich) (33 μM) was applied during the whole experiment.

Supplementary data

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

Acknowledgements

We gratefully acknowledge Geri Kreitzer for her generous and invaluable advice in reference to the study of kinesins and non-centrosomal microtubules in MDCK cells. We gratefully acknowledge Keith Mostov, Tim McGraw, Ching-Hwa Sung, Manoocher Soleimani and Ira Mellman for valuable reagents. We thank Drs Fred Maxfield, Tim McGraw and Marino Zerial for reading the manuscript and insightful suggestions. This work was supported by NIH grants GM54107 and EY08538, by the Research to Prevent Blindness Foundation and by the Dyson Foundation.


Conflict of interest

The authors declare that they have no conflict of interest.

References


proteins in recycling and biosynthetic routes of MDCK cells. *Proc Natl Acad Sci USA* 104: 1564–1569


A new pathway for mitochondrial quality control: mitochondrial-derived vesicles

Ayumu Sugiura, Gian-Luca McLelland, Edward A Fon & Heidi M McBride*

Abstract

The last decade has been marked by tremendous progress in our understanding of the cell biology of mitochondria, with the identification of molecules and mechanisms that regulate their fusion, fission, motility, and the architectural transitions within the inner membrane. More importantly, the manipulation of these machineries in tissues has provided links between mitochondrial dynamics and physiology. Indeed, just as the proteins required for fusion and fission were identified, they were quickly linked to both rare and common human diseases. This highlighted the critical importance of this emerging field to medicine, with new hopes of finding drugable targets for numerous pathologies, from neurodegenerative diseases to inflammation and cancer. In the midst of these exciting new discoveries, an unexpected new aspect of mitochondrial cell biology has been uncovered; the generation of small vesicular carriers that transport mitochondrial proteins and lipids to other intracellular organelles. These mitochondrial-derived vesicles (MDVs) were first found to transport a mitochondrial outer membrane protein MAPL to a subpopulation of peroxisomes. However, other MDVs did not target peroxisomes and instead fused with the late endosome, or multivesicular body. The Parkinson’s disease-associated proteins Vps35, Parkin, and PINK1 are involved in the biogenesis of a subset of these MDVs, linking this novel trafficking pathway to human disease. In this review, we outline what has been learned about the mechanisms and functional importance of MDV transport and speculate on the greater impact of these pathways in cellular physiology.

Keywords mitochondria; Parkin; PINK1; quality control; vesicle transport

Review

Introduction

Mitochondria are very complex organelles, housing hundreds of biochemical reactions from energy production to amino acid and lipid synthesis, to hormone production. These biochemical reactions involve substrates and products that flow between the many organelles within the cell. Rather than metabolite shuttling through free diffusion mechanisms, there is increasing evidence that direct interorganelar contacts are required. For example, elemental iron uptake into the mitochondria has been shown to require “kiss-and-run” contacts between the endosome and mitochondria (Zhang et al., 2005; Sheftel et al., 2007). Direct contacts between mitochondria and lipid droplets and peroxisomes are thought to facilitate fatty acid transport. The most advanced understanding of these contacts is between the ER and the mitochondria. It has long been known that ER is the source of lipids for mitochondrial biogenesis (Shiao et al., 1995) and that these contacts are important for cellular calcium homeostasis (Rizzuto et al., 1998). More recently, it was discovered that ER wrapping around the mitochondria marks the sites for mitochondrial division. A molecular understanding of these contacts has been advanced through studies in yeast and mammalian models (Csordas et al., 1999; de Brito & Scorrano, 2008; Kornmann et al., 2009) and is reviewed elsewhere. It is now clear that there is extensive biochemical cross talk between organelles, but the mechanisms are only beginning to emerge (Sheftel et al., 2007; Zehmer et al., 2009; Rowland & Voeltz, 2012; Mesmin et al., 2013).

This review will outline the emerging role of vesicular transport as another means of interorganelar communication. Mitochondrial-derived vesicles (MDVs) are generated through the selective incorporation of protein cargoes, which can be limited to the outer membrane, or can include outer, inner membrane, and matrix content, as illustrated in Fig 1 (Neuspiel et al., 2008; Soubannier et al., 2012a,b). Ultrastructural analysis revealed their size to be relatively uniform, between 70 and 150 nm, and their scission does not require the established mitochondrial fission GTPase Drp1 (Neuspiel et al., 2008; Soubannier et al., 2012a,b). Two distinct fates were identified for MDVs, with their targeting either to the late endosome/multivesicular body for degradation (Soubannier et al., 2012a), or to a subpopulation of peroxisomes (Neuspiel et al., 2008). Although this area of research is just emerging, this review will outline the molecular details of cargo selection, vesicle formation, and delivery, as well as the established and predicted impact of these pathways in cellular physiology.

The selection of cargo for transport

Mitochondrial-vesicle transport carries cargo to peroxisomes and lysosomes. Cargo destined for the lysosomes is ultimately degraded (Soubannier et al., 2012a), and in vitro studies have shown

*Corresponding author. Tel: +1 514 398 1808; E-mail: heidi.mcbride@mcgill.ca
enrichment of oxidized proteins within MDVs (Soubbannier et al., 2012b). However, the purpose of vesicle delivery to the peroxisomes is unclear (Mohanty & McBride, 2013). Currently, only one protein is known to traffic to the peroxisomes, a membrane anchored protein ligase called MAPL (also called MULAN, MUL1, GIDE and HADES) (Neuspiel et al., 2008; Braschi et al., 2009). With just one known cargo, it is difficult to predict the mechanisms and principles that govern cargo selection. However, we can look to evolution to help generate testable, working hypotheses. We will first consider the mechanisms of cargo selection based on each target destination separately.

**Cargo selection for transport to lysosomes** There are two primary pieces of evidence that contribute to our understanding of the nature of the cargo en route to the late endosome/lysosome. Firstly, MDVs generated in vitro from isolated mitochondria were shown to be enriched in oxidized protein, in a process that was stimulated by mitochondrial stress (Soubbannier et al., 2012b). This reconstitution of MDV formation further revealed a selective incorporation of protein cargo based on the nature of the mitochondrial stress induced. For example, the generation of ROS in the reaction with xanthine oxidase/xanthine led to a stimulation of MDVs carrying the outer membrane pore protein VDAC, but generation of ROS within the mitochondria, upon addition of a complex III inhibitor antimycin A, led to MDVs carrying the complex III subunit core2, without any enrichment in VDAC (Soubbannier et al., 2012b). These data suggest that potentially any cargo could be included within MDVs; assuming that they are first oxidized, which would “damage” the complex. This also suggests that oxidation may trigger aggregation or oligomerization, acting as a seed to initiate membrane curvature from the inside.

The process of MDV formation has likely been conserved from archaeabacteria, the mitochondrion’s ancestors (Deatherage & Cookson, 2012). So, are there clues as to the cargo selection mechanisms within these ancient systems? All gram-negative bacteria, including *Archaeb* strains, shed vesicles, from those living within the soil to infectious strains like *Helicobacter pylori*, causing ulcers, or *Treponema pallidum*, the cause of syphillis (Kulp & Kuehn, 2010; Bonnington & Kuehn, 2014). These bacterial strains bud a variety of vesicles carrying specific cargoes, with unique tasks from the transport of virulence factors, or peptides that arrest the host cell cycle, to the generation of a biofilm. In addition, the quantity of bacterial protein incorporation into vesicles ranges from 0.1% to 8–12%, showing a remarkable >100-fold variation in cargo incorporation (Soubbannier et al., 2012b; Bonnington & Kuehn, 2014). Environmental changes in pH or nutrients are often a signal for bacterial vesicle secretion; however, the molecular mechanisms responsible for such varied cargo selection are not well understood (Deatherage & Cookson, 2012). Given the diversity and complexity of bacterial vesicle formation, we consider it unlikely that a single evolutionary set of machinery could be mapped to MDVs from the ancestral mechanisms of shedding. However, the utility of vesicles in all membrane systems has been demonstrated and mitochondria are no exception. Indeed, the lessons from bacteria help to frame our understanding of the use of vesicles as a highly selective way to sort mitochondrial proteins.

The second important finding was that the generation of MDVs destined for lysosomes required the protein kinase PINK1 and the cytosolic ubiquitin E3 ligase Parkin (McLelland et al., 2014). PINK1 and Parkin are both mutated in familial cases of Parkinson’s disease (PD) (Trinh & Farrer, 2013) and were initially shown to act in a common pathway in mitochondrial quality control in *Drosophila* models of PD (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). More recent work has shown that PINK1 is targeted to the mitochondria but is normally degraded very rapidly. During import, PINK1 is first cleaved by the matrix processing peptidases and PARL (Jin et al., 2010; Greene et al., 2012); however, almost all of the cleaved PINK1 is then released from the import channel and degraded in the cytosol through the N-end rule proteolytic pathway (Kondapalli et al., 2012; Lazaro et al., 2012; Yamano & Youle, 2013). Upon mitochondrial depolarization, the import machinery is inactivated and PINK1 becomes trapped either within the import channel or becomes anchored to the mitochondrial outer membrane near the import channel (Greene et al., 2012; Lazaro et al., 2012). This exposes the kinase domain to the cytosol where it phosphorylates ubiquitin and Parkin, leading to stable Parkin recruitment and activation at the mitochondrial surface (Kim et al., 2008; Shibak-Fukushima et al., 2012; Iguchi et al., 2013; Kane et al., 2014; Kazlauskaite et al., 2014). Parkin ubiquitinates a series of proteins on the mitochondrial surface, which are then recognized by autophagic adaptor proteins and delivered to the autophagosome (Narendra et al., 2008; Gegg et al., 2010; Lee et al., 2010; Matsuda et al., 2010; Tanaka et al., 2010; Chan et al., 2011; Chen & Dorn, 2013; Sarraf et al., 2013).

Given that PINK1 and Parkin are also required for MDV transport, we predict that the same mechanisms apply, but at a much more localized level (see model, Fig 2). The import channels are spatially restricted upon the mitochondrial surface, as shown with super-resolution microscopy or immunoelectron microscopy (Wurm et al., 2011). Protein misfolding in the matrix was recently shown to trigger mitophagy after long incubations (Jin & Youle, 2013), without any loss of electrochemical potential. Therefore, we consider that local protein aggregation at the import site, perhaps due to local oxidative damage, complex assembly defects, would block the import process. Should the matrix chaperones become saturated, or cardiolipin become oxidized locally, then the inner membrane import channel may fail. Cardiolipin oxidizes to phosphatidic acid, a lipid known to alter membrane curvature (Yurkova et al., 2008; Donaldson, 2009; Horvath & Daum, 2013), and may help initiate the outward bending of the membrane. Upon complete depolarization or organelle dysfunction, the mechanism may switch from a local removal of a “patch” of mitochondrial content to the global arrest of PINK1 in all import channels, activation of the autophagic machinery, and entire engulfment of the organelle. This “patch” may not be strictly cargo selective, since whatever aggregated or oxidized proteins and lipids reside in proximity to an arrested import channel would be ejected. Supporting this concept, the kinetic analysis of events following treatment with antimycin A revealed the generation of MDVs at an early stage of ROS production, while global mitochondrial depolarization led to the kinetically slower process of mitophagy (McLelland et al., 2014). This indicates that MDVs are likely a first round of defense for the mitochondria to eject damaged proteins in order to avoid the complete failure of the organelle. This first response does not require the activation of autophagy machinery, as it occurs in the absence of Atg5, Rab9, or beclin (Soubbannier et al., 2012a; McLelland et al., 2014).
A new pathway for mitochondrial quality control

Ayumu Sugiura et al.

The EMBO Journal

Vol 33 | No 19 | 2014

© 2014 The Authors

The EMBO Journal

A new pathway for mitochondrial quality control

Ayumu Sugiura et al.

The EMBO Journal

Vol 33 | No 19 | 2014

© 2014 The Authors

A second argument in favor of the import channel acting as a sentinel for MDV formation is that import channels are restricted to the boundary membranes, where the inner and outer membrane are in close apposition to thread precursor proteins into the matrix and inner membrane. This explains how the two membranes may bud out together, since they would be locked in place by the arrested PINK1 precursor. It also lends insight into why PINK1 and Parkin are not required for the generation of MDVs that carry only outer membrane.

Figure 2. Working hypothesis for vesicle initiation by PINK1 and Parkin.

(A) Immunogold staining of endogenous Tom20 within COS7 cells reveals the regular spacing of the import channels indicated by arrowheads. Note the close tethering of three multivesicular bodies to the mitochondria.

(B) An illustration of our working hypothesis of PINK1/Parkin-mediated MDV formation. In Step 1, unfolded, oxidized proteins within matrix, triggered by ROS or failure to assemble, leads to protein aggregation (blue). Oxidation of cardiolipin will generate PA, contributing to altered membrane curvature. In Step 2, protein aggregates may saturate chaperones, leading to a very localized failure to import at an individual channel. In addition, local oxidation of cardiolipin would further interfere with import channels. PINK1, which is rapidly imported, would then accumulate at these failed import channels. In Step 3, PINK1 phosphorylates both ubiquitin and the ubiquitin-like domain of Parkin, stabilizing the recruitment of activated Parkin. The ubiquitination activity of Parkin is required to generate MDVs, suggesting that domains on the surface may be cleared. In Step 4, a vesicle is formed and released in a process that will certainly involve a number of unidentified proteins. Future studies are needed to test this hypothesis and uncover the details governing the generation of MDVs.

Figure 1. Summary of MDVs cargo variability.

Immunofluorescent and EM images illustrate the diversity of cargo-selected MDVs. Immunofluorescent staining of Tom20 (an outer membrane protein) and pyruvate dehydrogenase (PDH, matrix protein) reveals a number of cargo-selected vesicular structures lying outside of the mitochondria (top left panels, circles versus arrowheads).

Although Tom20 is absent from PDH-positive structures (arrowheads), EM and biochemical experiments confirm that these vesicles are double membrane bound. An example is shown to the left where both membranes are seen within the vesicle emerging from the intact mitochondria [with permission from Soubannier et al. (2012b)]. Similar cargo selectivity is seen for MDVs carrying MAPL that target the peroxisomes [top right panel of immunofluorescent images, taken with permission from Neuspiel et al. (2008)]. We also observe single membrane MDVs derived from just the outer mitochondrial membrane (EM panel on right side). Bottom electron microscopic pictures show MDVs containing Tom20 labeled by immunogold particles enter the multivesicular body [taken with permission from Soubannier et al. (2012a)]. Scale bars in EM pictures represent 100 nm.
A second argument in favor of the import channel acting as a sentinel for MDV formation is that import channels are restricted to the boundary membranes, where the inner and outer membrane are in close apposition to thread precursor proteins into the matrix and inner membrane. This explains how the two membranes may bud out together, since they would be locked in place by the arrested PINK1 precursor. It also lends insight into why PINK1 and Parkin are not required for the generation of MDVs that carry only outer

Figure 1. Summary of MDVs cargo variability. Immunofluorescent and EM images illustrate the diversity of cargo-selected MDVs. Immunofluorescent staining of Tom20 (an outer membrane protein) and pyruvate dehydrogenase (PDH, matrix protein) reveals a number of cargo-selected vesicular structures lying outside of the mitochondria (top left panels, circles versus arrowheads). Although Tom20 is absent from PDH-positive structures (arrowheads), EM and biochemical experiments confirm that these vesicles are double membrane bound. An example is shown to the left where both membranes are seen within the vesicle emerging from the intact mitochondria (with permission from Soubannier et al. [2012b]). Similar cargo selectivity is seen for MDVs carrying MAPL that target the peroxisomes (top right panel of immunofluorescent images, taken with permission from Neuspiel et al. [2008]). We also observe single membrane MDVs derived from just the outer mitochondrial membrane (EM panel on right side). Bottom electron microscopic pictures show MDVs containing Tom20 labeled by immunogold particles enter the multivesicular body (taken with permission from Soubannier et al. [2012a]). Scale bars in EM pictures represent 100 nm.

Figure 2. Working hypothesis for vesicle initiation by PINK1 and Parkin. (A) Immunogold staining of endogenous Tom20 within COS7 cells reveals the regular spacing of the import channels indicated by arrowheads. Note the close tethering of three multivesicular bodies to the mitochondria. (B) An illustration of our working hypothesis of PINK1/Parkin-mediated MDV formation. In Step 1, unfolded, oxidized proteins within matrix, triggered by ROS or failure to assemble, leads to protein aggregation (blue). Oxidation of cardiolipin will generate PA, contributing to altered membrane curvature. In Step 2, protein aggregates may saturate chaperones, leading to a very localized failure to import at an individual channel. In addition, local oxidation of cardiolipin would further interfere with import channels. PINK1, which is rapidly imported, would then accumulate at these failed import channels. In Step 3, PINK1 phosphorylates both ubiquitin and the ubiquitin-like domain of Parkin, stabilizing the recruitment of activated Parkin. The ubiquitination activity of Parkin is required to generate MDVs, suggesting that domains on the surface may be cleared. In Step 4, a vesicle is formed and released in a process that will certainly involve a number of unidentified proteins. Future studies are needed to test this hypothesis and uncover the details governing the generation of MDVs.
membrane proteins like the import receptor Tom20. Ultrastructural analysis of mitochondrial single membrane vesicles reveal a more pleotropic appearance, rather like “blebs” than true, well-constructed vesicles (Fig 1, Soubannier et al, 2012b). The trigger for these vesicles may more closely mirror the bacterial mechanisms of outer membrane vesicle release, the mechanisms of which remain unclear. Indeed, despite the relatively greater abundance of these Tom20 outer membrane vesicles compared to, for example, MDVs carrying matrix pyruvate dehydrogenase that lack Tom20, to date no protein machineries required for their biogenesis have been identified.

A role for Parkin in vesicle trafficking has been shown previously. In receptor-mediated endocytosis, a ubiquitin-interacting motif (UIM) within the adaptor protein Eps15 was shown to bind the ubiquitin-like domain (UBL) of Parkin (Fallon et al, 2006). This led to the monoubiquitination of Eps15 and inhibition of its capacity to recruit the endocytic machinery, thereby regulating its function as an adaptor for endocytosis of the EGF receptor (EGFR). In this way, by delaying EGFR endocytosis and degradation, Parkin can enhance signaling downstream of the receptor. Parkin was also shown to bind and monoubiquitinate the endocytic BAR domain protein endophilin A via a Ubl-SH3 interaction (Trempe et al, 2009).

As BAR domains are involved in membrane remodeling and curvature, this finding further links Parkin to vesicle budding and trafficking machinery. It is unclear what the signal is to recruit and activate Parkin at the cell surface as it is unlikely to be PINK1, which is constitutively targeted to mitochondria. However, very recent data have shown that PINK1, upon stabilization at the mitochondrial outer membrane, phosphorylates ubiquitin at position 56S (Kane et al, 2014; Kazlauskaite et al, 2014; Koyano et al, 2014). Three independent studies demonstrated that phosphorylated ubiquitin efficiently activated Parkin ubiquitin ligase activity at the mitochondrial surface in acute settings of mitochondrial uncoupling. In this situation, there was a nearly stoichiometric phosphorylation of cellular ubiquitin, which is likely to reach other cellular ubiquitin targets. In this way, the generation of phosphor-ubiquitin at the mitochondrial surface could act as a signaling mechanism for a global cellular response to mitochondrial stress. Regardless of the mechanisms by which Parkin is activated in endocytosis, the data indicate that Parkin may have a multifaceted role in vesicle transport, at the plasma membrane, mitochondrial surface, and perhaps elsewhere. It will be particularly interesting to determine whether these or other adapters are involved in the membrane budding and trafficking involved in the biogenesis of the subset of MDVs involving PINK1 and Parkin at mitochondria. Thus, whereas Parkin clearly plays a role in mitophagy, it also has a steady-state role in the removal of selected, oxidized cargo in a pathway parallel to mitophagy.

Cargo selection for transport to peroxisomes

The fate of mitochondrial cargo transiting to the lysosome is to be degraded. However, it is much less obvious why there may be a need for vesicle transport to the peroxisomes. The only cargo identified to date transits to a subpopulation of peroxisomes, about 10–20% of the total peroxisomes in the cell (Neuspiel et al, 2008; Braschi et al, 2010). Immunogold analysis of MAPL-positive MDVs revealed the presence of two membranes (Neuspiel et al, 2008), leading us to consider that the cargo is not limited to outer membrane content. There is some information on the mechanisms of MAPL enrichment within peroxisome-bound MDVs. The retromer complex containing Vps35, Vps26, and Vps29 was identified as a MAPL binding partner in an affinity chromatography approach (Braschi et al, 2010). The retromer complex was first established as a coat-like complex that binds and enriches cargo into vesicles from the endosome for their return to the Golgi apparatus (Seaman et al, 1998; Arighi et al, 2004; Seaman, 2012). The retromer complex also binds to the sorting nexin family of proteins that contain a PX-BAR domain that facilitates membrane curvature required for vesicle formation. More recent experiments reveal a much broader role for the retromer complex in many transport pathways, where specificity is granted through the combinatorial use of different sorting nexin members, and variants of the retromer subunits (Rojas et al, 2007; Collins et al, 2008; Cullen & Korswagen, 2012). In each case, the Vps35 subunit of the retromer binds to cargo tails, hinting that transport to the peroxisome will be more signal specific compared with the mechanisms of transport to the lysosome. Silencing Vps35 blocked the delivery of MAPL to peroxisomes, confirming the functional requirement for this complex in MDV transport (Seaman, 2012). MAPL contains ubiquitin and SUMO E3 ligase activities within the cytosolic domain (Braschi et al, 2009), however mutations in the RING finger domain did not alter the delivery to the peroxisome (Neuspiel et al, 2008; Braschi et al, 2010), indicating that the SUMOylation/ubiquitination activity of MAPL are not mechanistically required for MDV formation. At this time, we consider that MAPL constitutes vesicle cargo that does not function in the generation of peroxisome-bound vesicles. Clearly, there is a great deal of work remaining to elucidate the extent of cargo incorporation and the role of the retromer complex in this pathway.

Vps35 participates in a variety of transport pathways throughout the cell. However, with mutations in Vps35 being recently linked to PD and Alzheimer’s disease (Vilarino-Guell et al, 2011; Zimprich et al, 2011), its role in MDV transport has emerged as an intriguing functional arc that may link defects in Vps35 with mitochondrial dysfunction. Future work will determine whether an alteration in cargo delivery to peroxisomes may contribute to PD.

Mechanisms of MDV transport and delivery

As described above, we have identified three factors required for the generation of at least a subset of MDVs; those carrying matrix content for delivery to the lysosome (PINK1 and Parkin), and MDVs destined for the peroxisome (retromer complex). However, if we look to other vesicle transport paradigms, it is apparent that this is likely the tip of the iceberg. In addition to cargo selection mechanisms, MDV formation will require machineries that facilitate membrane curvature, potential coat complexes, incorporation of fusion machinery, and motility factors. MDVs are formed in the absence of the mitochondrial dynamin GTPase Drp1, indicating additional mechanisms are also required for the final scission event (Neuspiel et al, 2008; Soubannier et al, 2012a; McLelland et al, 2014). The independence of Drp1 is consistent with the diameter of the yeast mitochondrial dynamin (Dnm1) ring limited to 100 nm, which would be too large to constrict an MDV neck (Ingerman et al, 2005).
We may find clues as to the identity of MDV factors within the MitoCarta, an annotated map of the mitochondrial proteome (http://www.broadinstitute.org/pubs/MitoCarta/). For example, there are a number of vesicle-related proteins whose roles have not yet been characterized on the mitochondrial outer membrane. Two enzymes are predicted to modulate phosphatidylinositol phosphates (PIP) on the surface; PI(4,5)P₂ and splice variant of the PI(5)-phosphatase synaptojanin-2A (Nemoto et al., 2001). Although the presence of PIP-based microdomains on the mitochondria has not been studied intensively, PI(3)P domains were observed to form during mitophagy (Yang & Yang, 2013). PIP-related microdomains are known to recruit adaptor proteins that could favor membrane bending and vesicle generation (Mayinger, 2012). Consistent with adaptors that facilitate alterations in membrane curvature, another endophilin family member, endophilin B1 (Karbowiak et al., 2004; Takahashi et al., 2005, 2007), and a mitochondrial phospholipase D, MitoPLD (Choi et al., 2006; Huang et al., 2011), may also modulate membrane dynamics at the outer membrane. Endophilin B1 has been implicated in the binding of Bax (Pierrat et al., 2001), beclin (Takahashi et al., 2007) and in the process mitochondrial fission (Karbowiak et al., 2004). These lipid binding and modifying enzymes are all candidates for MDV transport machinery given the established roles for their activities in vesicle transport within the biosynthetic and endocytic pathways. Finally, several Rab GTPases have been shown to impact mitochondrial morphology, biogenesis or turnover, including Rab32, Rab11, Rab4, and Rab7 (Alto et al., 2002; Bui et al., 2010; Caza et al., 2013; Landry et al., 2014; Talaber et al., 2014; Yamano et al., 2014). Therefore, it would not be surprising if some of these small GTPases were involved in MDV transport as well.

It is also critical to learn how MDVs may fuse with their target organelle. A splice variant of VAMP1A, called VAMP1B, was identified in 1998 and contains a mitochondrial targeting sequence in place of the C-terminal tail anchor (Isenmann et al., 1998). VAMP1B is ubiquitously expressed, whereas VAMP1A variants are exclusive to neurons. The function of VAMP1B is currently unknown, but it is a prime candidate to mediate fusion events of MDVs with target organelles. Mitochondrial proteomic studies have not identified a t-SNARE or SNAP25 homologue, suggesting that the mitochondria may be unable to receive incoming vesicles.

Interestingly, a high-resolution proteome of a very divergent mitochondrion-related organelle, called a mitosome, from the parasite Giardia intestinalis was recently published (Jedelsky et al., 2011). Mitosomes have lost their mtDNA, as well as their capacity to respire, and have almost-unrecognizable import machinery. Their major role is in fact to generate iron sulfur clusters for distribution throughout the cell. Despite its divergence from a typical mitochondrion, the highly purified mitosome proteome included potential orthologues of the retromer component Vps35, an R-SNARE 3 (a v-SNARE) and VAP, a VAMP (vesicle associated membrane protein)-interacting protein (Jedelsky et al., 2011). Like mammalian mitochondria, Giardia mitosomes are also limited to v-SNAREs in the absence of t-SNAREs or SNAP orthologues. This provides a clue that even the simplest mitosome may sort cargo within vesicles for delivery within the cell, perhaps to distribute iron sulfur clusters to other organelles, or for degradation as we see in mammalian cells.

The physiological contribution of MDV transport to mitochondrial quality control

MDV transport to lysosomes adds a fourth mechanism to the paradigms of mitochondrial quality control. MDVs function alongside the actions of mitochondrial proteases, ubiquitin-mediated proteosomal degradation, and mitophagy. The unanswered question is to define the relative contributions and potential hierarchy of these mechanisms (Fig 3). Mitochondrial proteases degrade unfolded and oxidized proteins within the matrix and intermembrane space (Tatsuta & Langer, 2009). In yeast, an in vitro peptide export assay indicated that mitochondrial proteases degrade between 6–12% of proteins per hour, consistent with proteases as a front line of mitochondrial quality control (Augustin et al., 2005). It is also possible that proteases may trim down complexes and cargo, leaving more hydrophobic regions to be subsequently removed via MDVs, a possible example of the overlap among these pathways. Loss of mitochondrial proteases leads to various forms of neurodegeneration, including spastic paraplegia (Casari et al., 1998; Atrorino et al., 2003; Nolden et al., 2005). For example, mutations in AFG3L2, an m-AAA protease within the inner membrane, are responsible for spinocerebellar ataxia 28 (SCA28) (Di Bella et al., 2010). In addition, mitochondrial proteases are required for the processing of PINK1 (Greene et al., 2012), further supporting the interdependence between multiple quality control pathways.

Some outer membrane proteins are ubiquitinated and degraded by p97-dependent retrotranslocation and delivery to the cytosolic proteasome (Heo et al., 2010; Tanaka et al., 2010; Chan et al., 2011; Xu et al., 2011). This process efficiently removes surface proteins, which may be linked to quality control or to the selective removal of mitochondrial proteins in response to cellular signals (Neutner et al., 2007). For example, the anti-apoptotic Bcl-2 family protein Mcl-1 is ubiquitinated by MULE during apoptosis, and its removal facilitates Bax activation and cell death (Warr et al., 2005; Zhong et al., 2005).

Mitophagy is an important mechanism to remove entirely dysfunctional mitochondria, whether linked to global protein misfolding or depolarization (Youle & Narendra, 2011). But what is the contribution of MDVs to steady-state quality control, and how does this compare with mitophagy? Unfortunately, since PINK1 and Parkin are required for both pathways, gene editing or siRNA approaches will not allow us to easily answer this. Loss of these genes in flies leads to a loss of dopaminergic neurons and flight muscle defects, consistent with their importance in mitochondrial quality control (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). However, the loss of PINK1 or Parkin in mice leads to very mild phenotypes and a notable absence of neurodegeneration (Goldberg et al., 2003; Kitada et al., 2009). This hints at the existence of redundancies in both mitophagy and MDV formation in higher eukaryotes. Until we identify MDV-specific factors essential for their formation, we cannot determine the relative contributions in vivo. We do know that MDVs are released within 2–6 h following a mild stress like antimycin A, where mitophagy occurs between 12–24 h (McLelland et al., 2014). The prediction is that MDV formation protects the mitochondria from mitophagy by removing PINK1 and Parkin from each failed import channel. Any loss of MDV-specific machinery should therefore trigger premature mitophagy. Alternatively, the loss of MDVs may lead to increased global cellular...
damage since the mitochondria may need to deteriorate to an appropriate level in order to engage the mitophagy pathway. Deteriorating mitochondria would release increased levels of ROS and likely begin to consume cellular ATP in efforts to maintain their electrochemical potential.

Beyond a global consideration of MDVs acting upstream of mitophagy in quality control, there are a few clues that shed some light on the functional importance of MDV formation in mitochondrial homeostasis. Treating cultured cells with the lysosomal inhibitors bafilomycin A or pepstatin A/E-64D led to a significant accumulation of MDVs in the cytosol or within the multivesicular bodies (Soubannier et al., 2012a; McLelland et al., 2014). This demonstrated that MDV transport occurs in steady state, even in the absence of mitochondrial or redox-based stress. In addition, the amount of cargo released into MDVs using the in vitro budding assay was quantified, indicating that up to 4% of some proteins are ejected per hour (Soubannier et al., 2012b). While there are certainly limitations to these kinds of quantifications, it suggests that MDVs are transporting significant amounts of cargo in steady state.

In other approaches, a number of recent studies have quantified the half-lives of mitochondrial proteins under various conditions, from yeast to flies and mammalian tissues. The most striking observation from all of these studies is the extreme variety of half-lives of mitochondrial proteins—from minutes to hours. For example, a recent study in mice demonstrated a threefold range in the turnover rates of different complex I subunits, indicating that mitochondrial proteins within the same macromolecular complex have differing half-lives (Kim et al., 2012). This is consistent with the existence of multiple, overlapping pathways for mitochondrial protein degradation. In an elegant study in Drosophila by the Pallanck lab, the accumulation of cellular proteins in flies lacking the core autophagy protein Atg5, compared with those lacking Parkin or PINK1, was quantified by mass spectrometry (Vincow et al., 2013). In the absence of Atg5, many cellular proteins—including ones targeted to mitochondria—accumulated, indicating that Atg5 was required for their turnover. There was a great deal of overlap between the Atg5 and Parkin accumulated proteomes, consistent with them functioning along the same pathway in mitophagy. However, there was a distinct subset of highly hydrophobic proteins of the mitochondrial inner membrane that accumulated specifically in the absence of Parkin (Vincow et al., 2013). This indicates that Parkin plays an additional role the selective removal of these proteins, most likely through vesicular carriers.

In yeast, the Abeliovich lab recently confirmed the selective degradation of mitochondrial content using a similar approach (Abeliovich et al., 2013). In this case, they observed a selective accumulation of proteins in stationary phase yeast upon the loss of Dnm1, the functional homologue of the fission GTPase Drpl. The authors concluded that mitophagy results in the selective removal of damaged content at very different rates and that this process requires ongoing fusion and fission events. Exactly how the cargo would be sorted into two halves of a dividing mitochondrion remains unclear. The authors proposed a type of “percolation” of specific proteins into large domains, perhaps based on aggregation. These large domains would eventually be separated from the functional organelle by dynamin-mediated constriction (Abeliovich et al., 2013). On the other hand, it is formally possible that yeast mitochondria bud vesicles in a Dnm1-dependent manner, where the lateral segregation of cargo may occur in an analogous manner to MDV transport in mammalian systems. There is a great deal of

---

**Figure 3. Outline of the 4 pathways of mitochondrial quality control.**
A schematic diagram depicting the presence of mitochondrial proteases within the mitochondrial matrix and intermembrane space, which likely acts as a first line of defense against unfolded and oxidized soluble proteins. Outer membrane proteins are instead removed from the mitochondria through a retrotranslocation pathway following ubiquitination. Degradation of these proteins is completed within the cytosolic proteasome, similar to the ER-associated degradation pathway. We propose that the third line of defense is the removal of mitochondrial patches through the generation of MDVs, which transit to the late endosome. Only upon complete mitochondrial dysfunction, or upon a failure of most import channels would the entire organelle be targeted to the autophagosome. Different tissues and cellular states may rely on each of these mechanisms to a variable degree, making it important to understand the levels of redundancy and overlap among these pathways.
The field of mitochondrial quality control has exploded with the causal links to many degenerative diseases. So do any of the recent studies support a role for MDVs in quality control? The most common approach is to use the protonophore CCCP to globally depolarize mitochondria and induce Parkin-dependent mitophagy. However, recent studies are expanding the analysis to more physiological systems, including the unfolded protein response (Jin & Youle, 2013), laser-induced damage (Yang & Yang, 2013), and the manipulation of mitochondrial proteases that interfere with PINK1 processing, such as the matrix processing protease (MPP) (Greene et al., 2012). Indeed, modest knockdown of the catalytic subunit MPPβ leads to PINK1 accumulation, Parkin recruitment, and mitophagy, without the widespread effects seen with CCCP on protein import and mitochondrial function (Greene et al., 2012). Using a different approach, the Youle lab employed a non-cleavable mutant of ornithine carbamyl transferase (Jin & Youle, 2013), first used to induce the mitochondrial unfolded protein response by Nick Hoogenraad (Zhao et al., 2002). Cells expressing this construct accumulated PINK1-YFP at specific foci along the mitochondrial tubules (Jin & Youle, 2013), presumably reflecting sites of failed import. Parkin was recruited to these sites as well, and after extended accumulation of unfolded proteins, respiratory competent mitochondria were cleared by mitophagy. Similarly, foci of Parkin and PI(3)P were observed along mitochondria following laser ablation (Yang & Yang, 2013), and these small fragments were targeted for mitophagy. In all these studies, the authors did not explore whether they were actually imaging MDVs as an early, targeted response to the stress. Certainly, if the magnitude of the damage is great enough, mitophagy will ensue, as is commonly observed. Interestingly, two tail-anchored mitochondrial membrane proteins, FKBP38 and Bcl-2, were shown to translocate to the endoplasmic reticulum to escape CCCP-induced mitophagy (Saita et al., 2013). The mechanism for this relocalization is not known, but the authors suggested the possibility of vesicle-based transport prior to mitochondrial removal by mitophagy. As the size of MDVs is smaller than the wavelength of light, routine confocal microscope approaches cannot distinguish between a mitochondrial fragment (~400 nm diameter) and an MDV (~100 nm) (Neuspiel et al., 2008; Soubannier et al., 2012a). Antibody staining can reveal the selection of specific mitochondrial cargo in these structures, and silencing Drp1 can help address the question of fission vs. budding. In the end, ultrastructural analysis is critical to directly visualize these events, something lacking from current studies. Some experimental considerations relevant to the analysis of cellular MDVs are listed in Box 1.

In order to ultimately define the functional contribution of MDV transport to mitochondrial quality control in vivo, we will continue to identify the core machinery required to generate and transport MDVs. We are currently utilizing the cell-free mitochondrial budding assay to generate vesicles in the presence of non-hydrolyzable GTP, for example, to stabilize potential coat proteins and other machinery. Mass spectrometry of isolated vesicles will help identify both the cargo and machinery. Lipidomics should lend insights into any lipid oxidation or enrichments that are likely to be very important cargoes as well. As the mechanisms gain in resolution, we will be able to directly assess the consequences on mitochondrial function and cell survival in different cell types and tissues.

The meaning of MDV transport to the peroxisome

Next to the ER, it can be argued that the peroxisome is the most closely linked organelle to the mitochondria (Mohanty & McBride, 2013). In mammalian cells, both organelles are responsible for the

---

**Box 1. Experimental considerations for MDV analysis**

- MDVs are visualized as small vesicular structures that show evidence of cargo selectivity. This is done using highly specific antibodies against endogenous mitochondrial proteins, or with a combination of transfected mitochondrial GFP-tagged constructs with antibodies to label a second or third mitochondrial protein. The limitation of antibody usage is the absolute dependence on the specificity and low or zero background signal. GFP-tagged and overexpressed proteins have not been efficient cargoes in general, perhaps because they are first targeted by proteases. We continue to evaluate mitochondrial content in MDVs and look to proteomics approaches for future analysis.

- To increase the ability to visualize MDVs, it is useful to minimize mitochondrial fragmentation by silencing the fission machinery, or expressing a dominant-negative Drp1. This results in hyperfused mitochondria, enabling the visualization of small, Drp-independent MDVs within the cell.

- To capture MDVs within the cytosol, prior to delivery to the lysosome, cells can be pre-treated with bafilomycin A. Alternatively, inhibition of lysosomal proteases with E64D/pepstatin A allows delivery to lysosomes, but blocks protein degradation. For transport to peroxisomes, the cargo is not degraded, so MAPL-positive MDVs en route to peroxisomes (or already within peroxisomes) is monitored by triple-labeling mitochondria, MAPL, and peroxisomes. We do not yet have the tools to block the fusion of MAPL-positive MDVs with peroxisomes and accumulate them within the cytosol.

- Our experience with immunofluorescence approaches indicates a requirement for high percentages of PFA (5-6%), added directly to cells at 37°C without a PBS wash. This hints that the lipid/protein ratio in MDVs is not easily crosslinked and may be lost with low concentrations of fixatives.

- The signal intensity of cargo within MDVs varies. Tom20-positive MDVs are brighter in Tom20 signal compared with Tom20 intensity within mitochondria, but PDH is less enriched in MDVs compared with the mitochondria. Given this variability, it is important to use an appropriate objective lens, preferably 100×, with the highest NA possible, at least a 1.4. Cameras for spinning confocal continue to improve, increasing the ability to visualize MDVs, and laser scanning confocal at high resolution also works well.

- Ultrastructural analysis is important to directly visualize MDVs within the cell using immunogold labeling. This is to confirm their size and differentiate them from Drp1-dependent fragments. The probabilities of capturing an unbudded MDV from the mitochondria is low unless cells are treated with stress agents like xanthine oxidase/xanthine. Overexpressing MAPL in cells led to a dramatic stabilization of unbounded structures, suggesting that the GFP tag interfered with efficient removal. With the ongoing identification of new machinery required to generate MDVs, their silencing should help capture MDVs in various stages of formation. Tomography of tissues from disease animal models should also help visualize MDVs emerging in situ at high resolution.
beta-oxidation of fatty acids, where peroxisomes selectively oxidize very long-chain fatty acids and perform alpha-oxidation reactions. Both organelles neutralize damaging oxidative by-products—peroxides (peroxisomes) or ROS (mitochondria)—and the biogenesis of both organelles is signaled by the peroxisome proliferator-activated receptor coactivator 1 alpha (PGC1α) pathway (Mohanty & McBride, 2013). The biogenesis and growth of both organelles is also coupled to the ER, which provides lipids to facilitate their growth. Finally, the organelle division machinery is shared, including the core fission GTPase Drp1 and its receptor Mff (Koch et al, 2003; Gandre-Babbe & van der Bliek, 2008). Indeed, membrane-anchored proteins like Mff are commonly localized to both peroxisomes and mitochondria. (Koch et al, 2005; Gandre-Babbe & van der Bliek, 2008). Most of these tail-anchored proteins appear to be imported into peroxisomes using the canonical, peroxisomal import machinery (Dellile & Schrader, 2008).

We identified MAPL as a SUMO E3 ligase containing two transmembrane domains, with a C-terminal RING domain exposed to the cytosol and a approximately 40 kDa intermembrane space domain of unknown function (Li et al, 2008; Neuspiel et al, 2008; Zhang et al, 2008; Braschi et al, 2009; Jung et al, 2011). This protein has a unique evolutionary phylogeny, with the intermembrane space and transmembrane domains being conserved within distant bacteria, as well as plants (Andrade-Navarro et al, 2009). MAPL promotes the sumoylation of Drp1, stabilizing its recruitment to the mitochondria and promoting fission (Neuspiel et al, 2008; Braschi et al, 2009).

If peroxisomes can import their own proteins, why would MAPL require such a complex vesicular transport pathway? We are actively pursuing an answer to this question, so will only speculate here. Peroxisomes grow and divide as autonomous organelles. However, unlike the mitochondria, which multiply exclusively as a result of the enlargement and fission of existing organelles, peroxisomes can also be generated de novo (Dimitrov et al, 2013). Current evidence, primarily in yeast, supports a role for the ER in the generation of new peroxisomal precursor vesicles that may mature (Hoepfner et al, 2005), or fuse to form a mature organelle (Lam et al, 2010). The population of “young” versus “mature” peroxisomes varies depending on the cell type and growth conditions. As a regulator of Drp1, MAPL is likely to participate in peroxisomal biogenesis and division. The silencing of MAPL does not phenocopy Drp1, so it is not essential for mitochondrial fission, rather it can promote and stabilize Drp1 recruitment. It may be that MAPL is targeted to “young” or newly formed peroxisomes, which may have a higher rate of fission, or different regulatory mechanisms than the “older”, more mature peroxisomes.

A second reason to consider that MAPL-containing MDVs would target “young” peroxisomes is that old peroxisomes cannot fuse, only the ER-derived pre-peroxisomes are thought to be fusogenic (Dimitrov et al, 2013). Therefore, MDVs may deliver their cargo to the pool of nascent peroxisomes, and this cargo exchange may be necessary for peroxisomal maturation. As these peroxisomes grow and divide, MAPL would become diluted, or perhaps selectively degraded in some way. From an evolutionary perspective, it is interesting that most of the peroxisomal enzymes appear to derive from an archaeabacterial origin (Gabaldon et al, 2006). This has led some to suggest that peroxisomes originated as specialized mitochondria responsible for the breakdown of very long-chain fatty acids (and a number of other biochemical processes) (Speijer, 2014). Perhaps, this ancient vesicular transport pathway allowed the selective segregation of potentially damaging (or highly ROS generating) mitochondrial pathways to a newly formed organelle within the primitive eukaryotic cell.

Supporting the idea that the mitochondria contribute to the generation of peroxisomes de novo is the fact that many peroxisomal membrane proteins default to the mitochondrial outer membrane in patient fibroblast cells lacking peroxisomes (Sacksteder et al, 2000; South et al, 2000; Kim et al, 2006; Toro et al, 2009). This is not an exclusive pathway, as other peroxins target the ER under these conditions (Kim et al, 2006; Toro et al, 2009; Yonekawa et al, 2011), as in yeast. To date, this mitochondrial default pathway in mammalian cells has not been considered physiologically meaningful. However, this observation is consistent with the mitochondria as a contributor to peroxisomal biogenesis. Indeed, a recent study from the Erdman laboratory showed that a yeast mutant strain lacking Pex3 was rescued by ectopically targeting Pex3 to the mitochondria, resulting in the biogenesis of functional peroxisomes in cells that previously lacked these organelles (Rucktaschel et al, 2010). This indicates that the machinery exists to generate peroxisomes from mitochondria, even in yeast. We are currently exploring whether the retromer subunit Vps35 may bind to mitochondrial-localized peroxins like Pex3. Upon transfection of Pex3-GFP, some cells show high levels of mitochondrial targeting even when peroxisomes are functioning normally (Kim et al, 2006). It is also possible to test whether the retromer is required to generate new peroxisomes in patient fibroblasts upon rescue with the missing peroxin. Approaches like this should help define the functional contribution of MDV transport to peroxisomes.

What mitochondrial cargo do peroxisomes require that cannot be simply imported directly from the cytosol? These organelles share many metabolites, including heme, which is generated from iron sulfur clusters in the mitochondria and required for peroxisomal enzymes like catalase (Lazarow & de Duve, 1973; Stehling et al, 2014). Phospholipids like phosphatidylethanolamine, which is used to generate plasmalogens (Braverman & Moser, 2012), and perhaps very long-chain fatty acids that may be misdirected to the mitochondria could be carried more easily within MDVs than via soluble, cytosolic intermediate. With the identification of the retromer complex as requisite for this vesicle transport pathway, we can dissect the functional consequences on peroxisomal function when Vps35 is lost (Braschi et al, 2010). For example, if heme is a cargo, then loss of Vps35 may lead to oxidative stress within peroxisomes through catalase dysfunction. This would be particularly dangerous for neurons, since it is established that patients suffering from peroxisomal disorders present with a primarily neurological phenotype (Powers, 2001). Patients suffering from Zellweger syndrome carry mutations in core peroxisomal import proteins and therefore lack peroxisomes. The primary manifestation of the disease is a loss of myelination and neuronal cell death through the accumulation of very long-chain fatty acids and ROS production (Barry & O’Keeffe, 2013). Although primarily a disease of infants, an analysis of peroxisomal dysfunction in age-related diseases like PD is currently gaining momentum (Fransen et al, 2013). In general terms, it is clear that the mitochondria and peroxisomes are intimately linked and that the dysfunction of one can lead to the dysfunction of the other (Baumgart et al, 2001).
A new pathway for mitochondrial quality control

Ayumu Sugiura et al

The EMBO Journal

An alternative fate for MAPL-containing MDVs may be to a specialized functional population of peroxisomes. For example, it is possible that peroxisomes may not all be identical; some may specialize in bile acid synthesis, some in long-chain beta-oxidation, and others in plasmalogen synthesis. It is difficult to envision how the import machinery would select for a subset of functional enzymes. On the other hand, the delivery of an MDV to a peroxisome would change the content by supplying metabolite substrates or enzymes, thereby actively generating a functionally distinct class of peroxisome. Although some evidence supports the existence of different populations of peroxisomes in isolated tissue, identified by altered buoyant densities (Schrader et al., 1994), the functional implications of these different pools have not been established. Again, once the fusion machinery and cargo are identified, we can directly test these hypotheses.

Other fates for MDVs?

The fate of mitochondrial cargo entering the late endosome/multivesicular body is primarily to be degraded, since the inhibition of lysosomal proteases led to an accumulation of mitochondrial content (Soubannier et al., 2012a; McLelland et al., 2014). However, there may be other fates of this cargo. The multivesicular body can be routed to the cell surface, where its limiting membrane can fuse with the plasma membrane (Raposo & Stoorvogel, 2013). This leads to the release of its internal contents—intraluminal vesicles (exosomes), which can contain protein and microRNAs, as well as vacuolar proteases—that can have broad-ranging effects on neighboring cells; from microRNA-based reprogramming to cancer cell migration (Raposo & Stoorvogel, 2013). Proteomic analysis of exosome content, compiled within the “Vesiclepedia” (http://www.microvesicles.org) indicates that up to 10% of secreted proteins are mitochondrial (Choi et al., 2013; Burke et al., 2014), although it is not known whether this is functionally significant. Autophagosomes can also be a source of unconventional secretion, which can include a subset of leaderless cytotoxic proteins (Zhang & Schekman, 2013), with some evidence of transit through the recycling endosome (Duran et al., 2010; Manjithaya et al., 2010). The most robust example of secreted autophagosomes occurs during erythrocyte development when the cytotoxic organelles are ejected to form the red cell (Ney, 2011). MDV transport to the late endosome in steady state would predict the presence of selected mitochondrial content in exosomes under most conditions (Fig. 2). Therefore, both mitophagy and MDV transport to the late endosome provide a means for the secretion of mitochondrial content from the cell.

The steady-state presence of mitochondrial content in exosomes may have some important physiological consequences. Under the conditions we have examined so far, we have shown that MDVs can contain at least three of the five complexes of the electron transport chain. Complexes III and IV both include subunits encoded by the mitochondrial DNA, which are translated with a formylated initiating methionine, conserved from bacterial translation (Kozak, 1983). The presence of extracellular formylated proteins or peptides can launch an immune response, whether of bacterial or mitochondrial origin (Zhang et al., 2010). This is because non-methylated DNA (Pollack et al., 1984) and formylated peptides bind and activate Toll-like receptor 9 leading to cytokine release and lymphocyte infiltration (Zhang et al., 2010). This pathway was shown to become activated within the endosome of cells lacking the lysosomal DNAse I (Oka et al., 2012). The subsequent accumulation of mtDNA within the autophagosome eventually escaped into the endosomal compartments, activating the receptors in the lumen of the endosome. Therefore, whether secreted through exosomes, or accumulated within the endosome, mitochondrial content has the potential to launch an inflammatory response.

Another possible link between MDVs, exosomes and disease may involve α-synuclein. α-Synuclein is an aggregation-prone cytosolic protein important in the pathogenesis of sporadic PD. Moreover, mutations in α-synuclein, like those in Parkin and PINK1, lead to a familial form of PD, albeit a dominant iteration of the disease (Klein & Westenberg, 2012). Secretion and cell-to-cell transfer of α-synuclein has been shown both in cells and in vivo (Lee et al., 2004, 2005, 2008a,b; Desplats et al., 2009; Luk et al., 2012a,b), and exosomes constitute a proposed method of propagation (Emmanouilidou et al., 2010; Kong et al., 2014; Lee et al., 2014). While a functional role for α-synuclein has been proposed in the assembly of presynaptic SNARE complexes (Chandra et al., 2005; Burre et al., 2010; Diao et al., 2013), α-synuclein has also been shown to function at mitochondria. A number of studies have identified α-synuclein as present either within mitochondria (Cole et al., 2008; Devi et al., 2008), or localized to mitochondrial-associated membranes of the ER (Guardia-Laguarta et al., 2014) and have implicated it in binding to anionic and cardiolipin-containing membranes (Nakamura et al., 2011; Zigoneanu et al., 2012; Diao et al., 2013) and in promoting Drp1-independent fission (a hallmark of MDV formation) in cells (Kamp et al., 2010; Nakamura et al., 2011). Thus, the incorporation of α-synuclein into multivesicular bodies via MDVs represents an intriguing candidate mechanism for its exosomal secretion, with additional implications for PD.

Final thoughts

With advances in the mechanistic characterization of MDV transport, we hope the field will be encouraged to carefully examine these structures within various physiological paradigms. There are many lines of evidence supporting the importance of MDV transport in mitochondrial cell biology; from an unbiased evolutionary consideration of the process (Kulp & Kuehn, 2010; Jedelsky et al., 2011), to their potential roles in peroxisomal function (Neupiel et al., 2008; Braschi et al., 2010) and mitochondrial quality control (Soubannier et al., 2012a,b; McLelland et al., 2014). MDVs can be observed by monitoring multiple mitochondrial markers by confocal microscopy, particularly in cells where Drp1 function is lost (Box 1). With the resulting hyperfused reticulum, these small vesicles are much more noticeable using high-resolution imaging techniques. Technically, there are still some limitations, primarily in the limited assortment of antibodies. There are approximately 1,000 proteins in the mitochondria, and we can only follow a handful using immunofluorescence approaches. In addition, we have noted that MDVs are somewhat fragile and not as easily fixed compared with the mitochondrial tubules (we routinely fix with 5–6% paraformaldehyde, for example). This likely reflects differences in lipid and protein composition within MDVs. In future studies, it will be important to examine the functional contribution of MDVs in
the physiology of specific tissues, particularly neurons, given the apparent links to Parkinson’s disease.

The mitochondria are the most recent eukaryotic organelle from which vesicle transport has been observed. However, there is some irony in the knowledge that this process has clearly been conserved from the organelle’s humble origins as archaeabacteria. It leads us to wonder what other fascinating and unexpected cellular pathways are waiting to be discovered as we dig deeper into the mechanisms and meaning of mitochondrial vesicles.

**Funding**

Canadian Institutes of Health Research (to EAF and HMM). Heart and Stroke Foundation of Ontario (to HMM)

**Author contributions**

HMM wrote the manuscript, AS, CLM and EAF contributed to the writing, the development of the concepts presented, and the generation of the figures.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


Desplats P, Lee HJ, Bae EJ, Patrick C, Rockenstein E, Crews L, Spencer B, Masliah E, Lee SJ (2009) Inclusion formation and neuronal cell death...
through neuron-to-neuron transmission of alpha-synuclein. Proc Natl Acad Sci USA 106: 13010 – 13015


Ayumu Sugiura et al A new pathway for mitochondrial quality control

The EMBO Journal The EMBO Journal  Vol 33 | No 19 | 2014 2153


Koch A, Yoon Y, Bonekamp NA, McNiven MA, Schrader M (2005) A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. Mol Biol Cell 16: 5077 – 5086


Yamano K, Youle R (2013) PINK1 is degraded through the N-end rule pathway. Autophagy 9: 1–12


