A novel assay reveals preferential binding between Rabs, kinesins, and specific endosomal subpopulations

Marvin Bentley, Helena Decker, Julie Luisi, and Gary Banker

Jungers Center for Neurosciences Research, Oregon Health & Science University, Portland, OR 97239

Identifying the proteins that regulate vesicle trafficking is a fundamental problem in cell biology. In this paper, we introduce a new assay that involves the expression of an FKBP12-rapamycin–binding domain–tagged candidate vesicle-binding protein, which can be inducibly linked to dynein or kinesin. Vesicles can be labeled by any convenient method. If the candidate protein binds the labeled vesicles, addition of the linker drug results in a predictable, highly distinctive change in vesicle localization. This assay generates robust and easily interpretable results that provide direct experimental evidence of binding between a candidate protein and the vesicle population of interest. We used this approach to compare the binding of Kinesin-3 family members with different endosomal populations. We found that KIF13A and KIF13B bind preferentially to early endosomes and that KIF1A and KIF1Bβ bind preferentially to late endosomes and lysosomes. This assay may have broad utility for identifying the trafficking proteins that bind to different vesicle populations.

Introduction

Organelles in the endomembrane system receive and dispatch vesicles in a highly regulated manner, which is mediated by a complex set of trafficking proteins: motors, SNAREs, tethers, Rabs, coat proteins, cargo adaptors, and others (Vale, 2003; Spang, 2008; Wickner and Schekman, 2008). Identifying the trafficking proteins that associate with different vesicle populations is an essential step in understanding the mechanisms that regulate vesicle trafficking. To accomplish this task, specific vesicle populations can be enriched by subcellular fractionation, immuno-isolation, or fluorescence sorting, and then the proteins present can be identified (Franzusoff et al., 1992; Takamori et al., 2006; Duclos et al., 2011; Zhang et al., 2011; Rhee et al., 2013). Two-color fluorescence microscopy is often used to confirm that a candidate protein binds the relevant vesicle population in vivo, in the appropriate biological context. However, this approach cannot always provide a definitive answer, as visual colocalization is often difficult to evaluate. Alternate approaches such as immuno-EM can be tedious.

Recently, we developed a new strategy to determine which members of a set of candidate kinesins associate with dendritically polarized vesicles in neurons (Jenkins et al., 2012). This "split kinesin" assay involves the expression of separate constructs encoding a kinesin tail fused to the FKBP12-rapamycin–binding (FRB) domain and a kinesin motor domain fused to the FKBP12 domain. These two domains can be inducibly assembled by adding a membrane permeant rapamycin analogue (Belshaw et al., 1996; Kapitein et al., 2010b; Robinson et al., 2010). The kinesin tail contains the cargo-binding domain and can bind vesicles, but it is incapable of influencing their movement because it lacks a motor domain. The kinesin motor domain is constitutively active and translocates into the axon but cannot move vesicles because it lacks a cargo-binding domain. Linking these two components together leads to a dramatic increase in vesicles entering the axon if and only if the expressed kinesin tail binds to the vesicles in question.

This assay offers several advantages. First, the induced change in vesicle trafficking is rapid and unmistakable. Second, the assay allows an unbiased evaluation of interactions between a given vesicle population and all relevant members of a family of trafficking proteins. Finally, the assay works well even if two different kinesins mediate the transport of a given organelle. Despite these advantages, the split-kinesin assay has limitations that restrict its applicability. First, the assay depends on the

Correspondence to Gary Banker: bankerg@ohsu.edu

H. Decker's present address is the Max Planck Florida Institute for Neuroscience, Jupiter, FL 33458.

Abbreviations used in this paper: BicD2, Bicaudal D2; FHA, forkhead associated; FKBP, FK506 binding protein; FRB, FKBP12-rapamycin binding; MAGUK, membrane-associated guanylate kinase; MTOC, microtubule-organizing center; tdTM, tandem dimer Tomato; TfR, transferrin receptor.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 208 No. 3 273–281
www.jcb.org/cgi/doi/10.1083/jcb.201408056

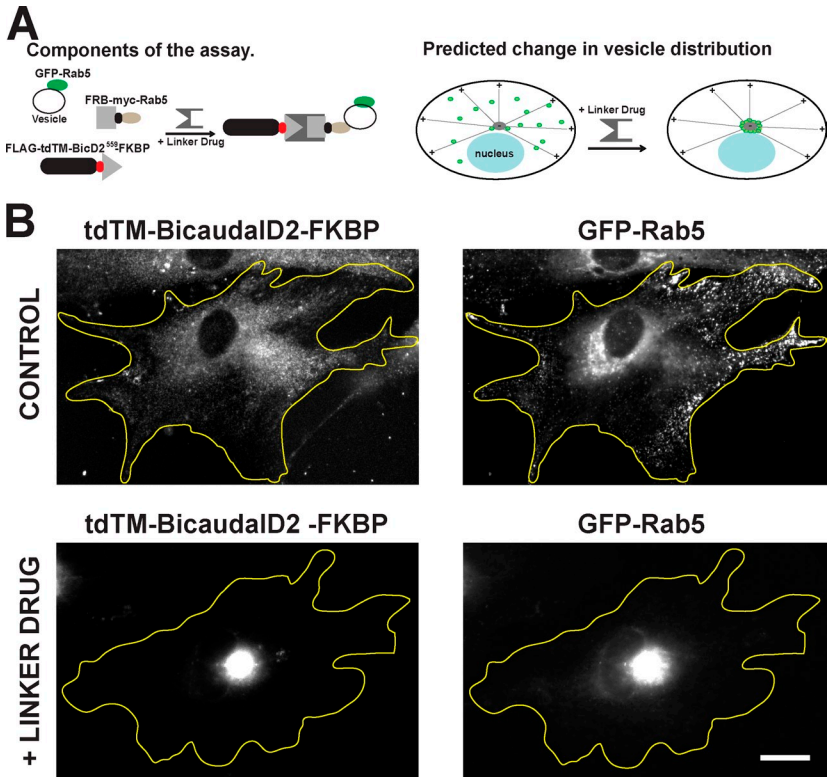

Figure 1. **Linking Rab5 to dynein by inducible dimerization causes early endosomes to accumulate in the cell center.** (A) As shown in the schematic, FRB-3myc-Rab5 and tdTM-BicD2^{594}-FKBP were coexpressed along with GFP-Rab5, a vesicle marker. After adding the linker drug (AP21967), the FRB and FKBP domains were fused together, linking the FRB-3myc-Rab5 to dynein. The second diagram shows the predicted redistribution of GFP-Rab5 vesicles to the minus end of microtubules (gray). (B) Representative images showing the distribution of tdTM-BicD2^{594}-FKBP and GFP-Rab5 in control cells and in cells treated with the linker drug. In control cells BicD2 was mostly soluble, with small aggregations throughout the cell. GFP-Rab5 vesicles were distributed through the cell. In treated cells, both GFP-Rab5 vesicles and BicD2 became concentrated in the center of the cell. The yellow lines outline the cell boundaries. Bar, 30 µm.

unique organization of neurons, which have spatially separate axonal and dendritic domains. Second, the assay requires live imaging to detect the increased vesicle flux that occurs after adding linker drug. This makes these experiments time consuming and technically challenging.

Here, we describe strategies for adapting this assay for use in other cell types, using a readout that does not require live imaging. In most cell types, microtubules originate from a central microtubule-organizing center (MTOC), and their plus ends extend toward the cell periphery. By expressing a candidate protein that can be linked to either a kinesin or dynein, it should be possible to mislocalize vesicles that bind that protein in a predictable manner. To move vesicles toward microtubule plus ends, we used the constitutively active kinesin KIF5C^{559} (Jenkins et al., 2012), and for transport toward the minus ends, we used the N-terminal fragment of Bicaudal D2 (BicD2), which can link vesicles to dynein (Kapitein et al., 2010a).

Using this approach, we were able to identify the Rabs and kinesins that bind to different endosomal populations, based on drug-induced mislocalization of vesicles when FRB-tagged candidate proteins were linked to active motors. This new assay generates robust and easily interpretable results and, in contrast to conventional, two-color localization experiments, provides direct experimental evidence that a candidate protein binds to a particular vesicle population.

Results and discussion

Linking Rabs to active motors results in mislocalization of endosomes

We set out to develop an assay in which inducible, motor-driven vesicle mislocalization serves as a readout to indicate which of a set of candidate trafficking proteins associates with a given vesicle population. Candidate proteins were tagged with FRB so that they could be linked to a constitutively active motor by rapamycin analogue-induced heterodimerization. We hypothesized that attaching dynein to vesicles would result in their accumulation near the MTOC. Previous work has shown that the localization of mitochondria or peroxisomes can be changed by adding a homotypic tether or by directly cross-linking constitutively active motors to integral membrane proteins present in these organelles (Sengupta et al., 2009; Kapitein et al., 2010a,b; van Spronsen et al., 2013). It is less clear whether linking motors to trafficking proteins that associate only transiently with the cytoplasmic surface of vesicles can produce a distinctive change in vesicle distribution.

As proof of principle, we asked whether linking an FRB-tagged Rab to an unregulated, constitutively active kinesin or dynein produces a distinctive change in the localization of the vesicles that bind that Rab. One potential advantage of directing vesicles toward the minus ends of microtubules is that they are likely to form a single aggregate near the cell center, which should provide a strong signal. We adapted the strategy developed by Kapitein et al., 2010a,b, who investigated dynein-driven peroxisome transport by inducibly linking BicD2, a dynein cargo adaptor, to peroxisomes (Dienstbier and Li, 2009; Kardon and Vale, 2009). We expressed a protein, tandem dimer Tomato (tdTM)–BicD2^{594}–FK506 binding protein (FKBP), consisting of FKBP fused to the dynein-binding fragment of BicD2, which was coexpressed with FRB-Rab5 (Fig. 1 A). In control cells, GFP-Rab5 vesicles were largely distributed in the cell periphery (Fig. 1 B). BicD2 was diffusely distributed throughout the cell; a small amount also appeared to be associated with vesicles (not apparent at the magnification shown in this figure).

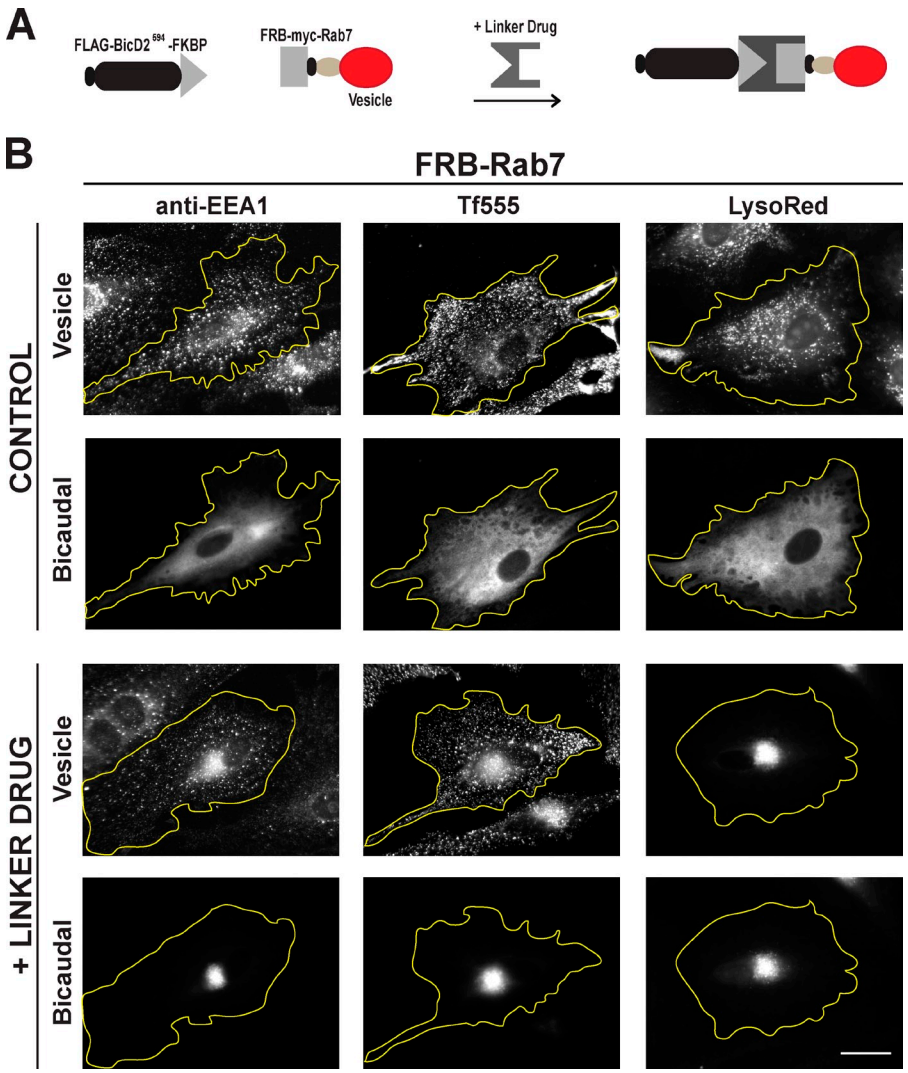

Figure 2. **Rab7 interacts primarily with late endosomes.** (A) A schematic showing the components of the assay. GFP-BicD2^{594}-FKBP and FRB-3myc-Rab7 were coexpressed. Vesicles were labeled by anti-EEA1 staining or by uptake of Tf555 or LysoTracker red. (B) Representative images showing the distribution of each vesicle population and the distribution of BicD2. In controls, EEA1- and Tf555-labeled vesicles were found throughout the cell, whereas LysoTracker red vesicles were more concentrated in the perinuclear region. BicD2 had a soluble distribution. In cells treated with the linker drug, the distribution of EEA1- and Tf555-labeled vesicles was largely unchanged. Vesicles labeled with LysoTracker red (LysoRed) were massively misdirected to the cell center. BicD2 also accumulated in the cell center. The yellow lines outline the cell boundaries. Bar, 30 µm.

In drug-treated cells, the localization of GFP-Rab5 vesicles was profoundly altered. Vesicles were found almost exclusively at the cell center, presumably because they were driven toward the MTOC by dynein. Virtually all of the BicD2 was also concentrated at the cell center, which suggests that molecules of BicD2 that dimerize with FRB-Rab5 remain attached to endosomal vesicles. A comparable experiment was conducted using a kinesin motor domain that was fused to FKBP (Vale et al., 1996; Friedman and Vale, 1999; Jacobson et al., 2006; Cai et al., 2009; Nakata et al., 2011). Endosomes were successfully misdirected to the cell periphery (Fig. S1), but the resulting change was not as obvious as that which resulted from linking vesicles to dynein.

In the experiment just described (and in those that follow), only about half of the transfected cells exhibited the profound changes in vesicle localization illustrated in Figs. 1 and S1. In the other cells, the distribution of endosomes and of BicD2 was no different than in controls. This is not surprising, because the assay depends on the coexpression of multiple proteins at levels appropriate to induce vesicle movement. Because the pattern of vesicle mislocalization induced by association with active motors was never observed in control cells and because this mislocalization requires that the FRB-tagged proteins bind to the labeled vesicles, we interpret positive results as strong evidence of vesicle binding, even if observed in only a fraction of the cells.

Evaluating the selectivity of Rab binding to early and late endosomes

We next asked whether the binding of different FRB-Rabs shows the expected selectivity for different endosome populations. We used two approaches to identify early endosomes: immunostaining to localize EEA1 and uptake of transferrin conjugated to Alexa Fluor 555 (Tf555). LysoTracker red was used to visualize late endosomes and lysosomes.

To determine which of these vesicles bound to Rab7, we expressed BicD2 and FRB-Rab7 (Fig. 2). In control cells, early endosomes were distributed throughout the cell, whereas late endosomes/lysosomes had a more perinuclear labeling pattern (Fig. 2 B). Cells expressing FRB-Rab7 and treated with the linker drug displayed some redistribution of the early endosome markers EEA1 and Tf555. However, most early endosomes

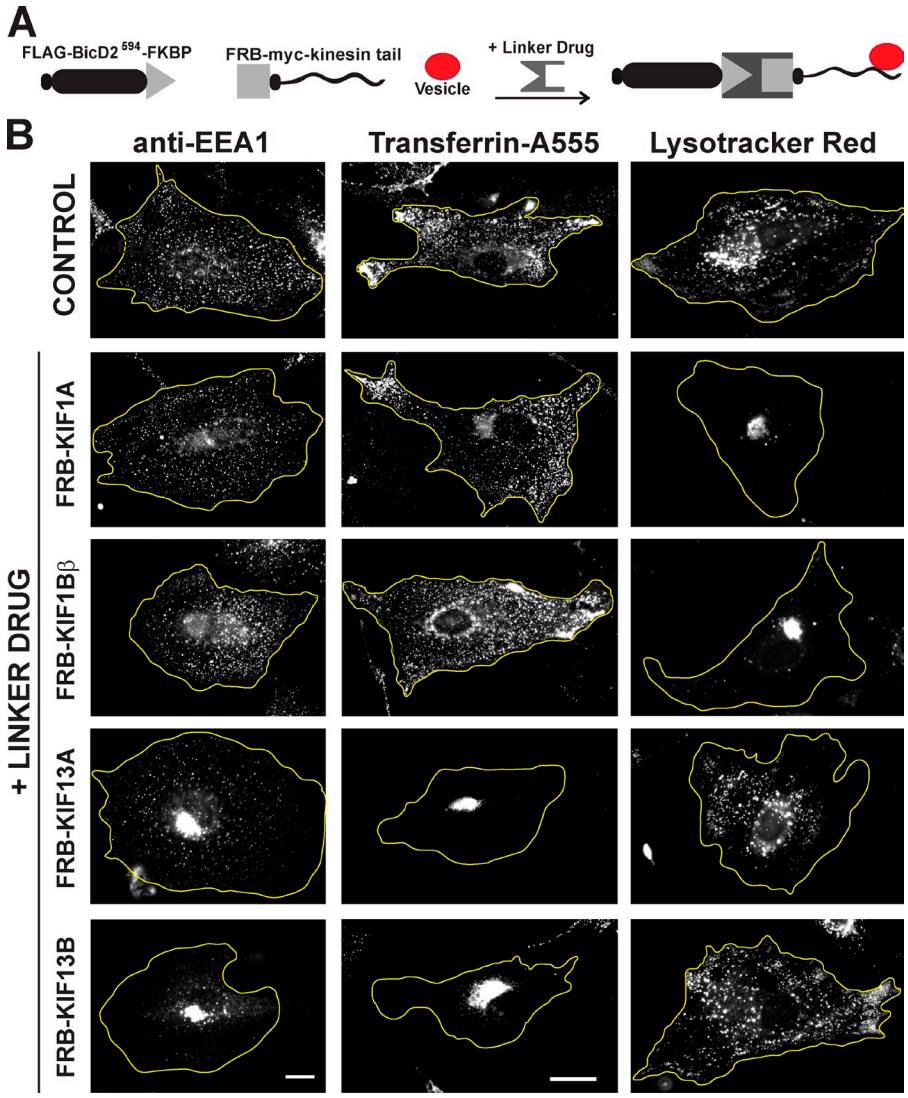

Figure 3. **Different kinesins bind to different endosomal populations.** (A) As shown in the schematic, GFP-BicD2^{594}-FKBP was expressed together with a series of FRB-tagged kinesin tails. Vesicles were labeled by anti-EEA1 staining or uptake of Tf*555* or LysoTracker red. (B) Representative cells showing the distribution of labeled vesicles. In controls, EEA1- and Tf*555*-labeled vesicles were found throughout the cell, whereas LysoTracker red vesicles were concentrated in the perinuclear region. In cells treated with the linker drug, EEA1- and Tf*555*-labeled vesicles were moved toward the cell center only in cells expressing FRB-3myc-KIF13A$_{tail}$ or FRB-3myc-KIF13B$_{tail}$. Vesicles labeled with LysoTracker red moved to the cell center only in cells expressing FRB-3myc-KIF1A$_{tail}$ or FRB-3myc-KIF1Bβ$_{tail}$. The control cell expressed FRB-3myc-KIF1A$_{tail}$ and was not exposed to the linker drug. The yellow lines outline the cell boundaries. Bars: (EEA1) 25 µm; (Tf*555* and LysoTracker red) 30 µm.

remained in the cell periphery, indicating that they did not bind FRB-Rab7. The mislocalization of some endosomes labeled with these markers is not surprising, as there is a period during the transition from early to late endosomes when vesicles carry both Rab5 and Rab7 (Rink et al., 2005; Poteryaev et al., 2010). In contrast, adding linker drug caused the accumulation of nearly all LysoTracker red vesicles in cells expressing FRB-Rab7; few if any vesicles could be detected outside of the aggregate. This indicates that FRB-Rab7 binds primarily to late endosomes and lysosomes in this assay, consistent with the known localization of this protein (Bucci et al., 2000). As expected from the results in Figs. 1 and S1, BicD2 also accumulated at the cell center when it was linked to vesicles. Similar experiments using the same vesicle markers and FRB-Rab5 showed preferential binding to early endosomes (Fig. S2). From these experiments, we conclude that this assay correctly reports the selective binding of FRB-Rab7 to late endosomes/lysosomes and FRB-Rab5 to early endosomes. The assay works equally well when endosomes are labeled in living cells or when they are visualized by immunostaining after the experiment has been completed.

Identifying the motor proteins associated with different endosomal populations

We next used this assay to investigate the kinesins that are present on different endosomal populations, using multiple approaches to label early and late endosomes (Figs. 3 and S2). We focused on motors of the Kinesin-3 family. Although some of these motors have been previously implicated in endosomal transport (Matsushita et al., 2004; Hoepfner et al., 2005; Blatner et al., 2007; Delevoye et al., 2009; Huckaba et al., 2011; Kanai et al., 2014), there has been no systematic study of whether different Kinesin-3's bind preferentially to different endosomes. We generated kinesin tails that included the cargo-binding domain but lacked the motor domain and the first coiled coil. These tails were fused to an N-terminal FRB-3myc. Each of these tails was then expressed together with FLAG-BicD2-FKBP to determine whether they bound to different endosomal populations (Fig. 3 A). We used the same three labels of endogenous vesicles as before. In control cells, each of the endosomal markers displayed its characteristic distribution, as described earlier. This distribution was unaffected by expression of any of the kinesin tails (Fig. 3 B).

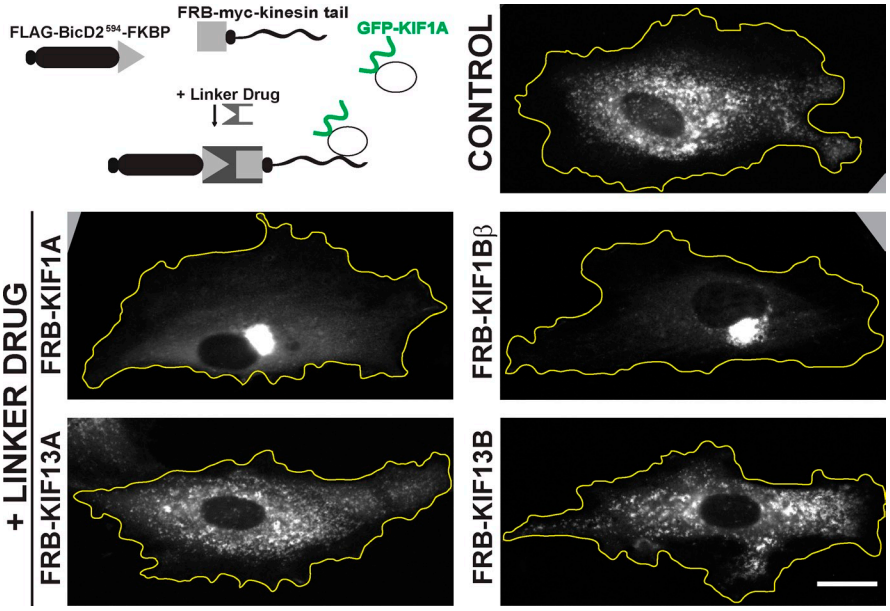

Figure 4. **Two different kinesins can bind the same vesicle.** As shown in the schematic, vesicles were labeled by expressing GFP-KIF1A$_{tail}$, which was coexpressed with tdTM-BicD2^{594}-FKBP and one of four different FRB-tagged kinesin tails. The remaining panels are representative images showing the distribution of vesicles labeled with GFP-KIF1A$_{tail}$. In control cells, GFP-KIF1A$_{tail}$ labeled small vesicles that were concentrated in the perinuclear region. After treatment with the linker drug, GFP-KIF1A$_{tail}$ vesicles accumulated in the cell center only in cells expressing FRB-3myc-KIF1A$_{tail}$ or FRB-3myc-KIF1Bβ$_{tail}$. The control cell expressed FRB-3myc-KIF1A$_{tail}$ but was not exposed to linker drug. The yellow lines outline the cell boundaries. Bar, 25 µm.

In cells expressing KIF1A$_{tail}$ or KIF1Bβ$_{tail}$ and treated with the linker drug, there was no change in distribution of early endosomes labeled with either Tf555 or EEA1, but there was a profound redistribution of the late endosome marker LysoTracker red. Labeled endosomes formed a compact cluster in the center of the cell; few vesicles could be detected elsewhere. In cells expressing KIF13A$_{tail}$ or KIF13B$_{tail}$ and treated with linker drug, there was no effect on the distribution of late endosomes/lysosomes, but the addition of linker drug caused EEA1 and Tf555 vesicles to aggregate in the cell center. This shows that different Kinesin-3 family members bind different endosomal populations; KIF1A and KIF1Bβ bind to late endosomes, whereas KIF13A and KIF13B bind early endosomes. These results were confirmed using a series of GFP-tagged proteins to label different endosomal populations (Fig. S3).

An individual vesicle can bind different kinesins

The results just described indicate that each endosomal population is capable of binding two different Kinesin-3 family members. This raises the question of whether different kinesins are found on individual endosomes or whether there are subpopulations of early and late endosomes that bind different kinesins. This assay can address this question by using one kinesin tail to label vesicles, then determining whether other FRB-tagged tails bind to the same vesicles. In the experiments shown in Fig. 4, GFP-KIF1A$_{tail}$ was used to label vesicles; it was coexpressed together with different FRB-kinesin tails and FLAG-BicD2-FKBP. In control cells, GFP-KIF1A$_{tail}$–labeled vesicles had a predominantly perinuclear distribution (Fig. 4 B). In cells expressing FRB-KIF1A$_{tail}$, GFP-KIF1A$_{tail}$–labeled vesicles aggregated at the cell center, as would be expected. In cells expressing FRB-KIF1Bβ$_{tail}$, GFP-KIF1A$_{tail}$ vesicles also aggregated at the cell center, demonstrating that KIF1A$_{tail}$ and KIF1Bβ$_{tail}$ bound to the same vesicles. Consistent with the results described earlier (Figs. 3 and S3), there was no misdirection of GFP-KIF1A$_{tail}$–labeled vesicles in cells expressing FRB-KIF13A$_{tail}$ or FRB-KIF13B$_{tail}$. These results show that a single vesicle can bind to both KIF1A and KIF1Bβ.

Using the assay to define the vesicle-binding domains of trafficking proteins

In principle, this assay could also be used to define the vesicle-binding domains of trafficking proteins. To evaluate this possibility, we attempted to define the domain of KIF13B that binds early endosomes (Fig. 5). The KIF13B tail contains a forkhead-associated (FHA) domain (residues 423–557) that binds centaurin-α (Tong et al., 2010) and a membrane-associated guanylate kinase (MAGUK) binding stalk (residues 607–831) that interacts with homologues of *Drosophila melanogaster* discs-large (hDlg; Hanada et al., 2000). Previous studies have implicated the interaction with centaurin-α and the interaction with hDlg as important for the binding of KIF13B to vesicles (Tong et al., 2010; Kanai et al., 2014). We generated an FRB-tagged fragment of the KIF13B tail that contains the FHA domain and the MAGUK binding stalk (KIF13B$^{442–831}_{tail}$). A second FRB-tagged construct contained the remainder of the tail (KIF13B$^{832–1,826}_{tail}$). These constructs were each coexpressed with transferrin receptor (TfR)–GFP and FLAG-BicD2^{594}-FKBP (Fig. 5 A). In control cells, TfR-GFP vesicles were distributed throughout the cell (Fig. 5 B). In cells that expressed KIF13B$^{442–831}_{tail}$ and were treated with the linker drug, there was no change in the localization of TfR-GFP vesicles. In cells expressing KIF13B$^{832–1,826}_{tail}$, addition of the linker drug resulted in a pronounced redistribution of TfR-GFP to the cell center. This experiment shows that residues 832–1,826 are sufficient to mediate binding of KIF13B tail to early endosomes and that neither the FHA domain nor the MAGUK binding stalk mediates this interaction.

Advantages and limitations of this new assay

The motor-driven vesicle mislocalization assay described here offers a new approach to evaluate protein–vesicle association that has several advantages compared with other methods.

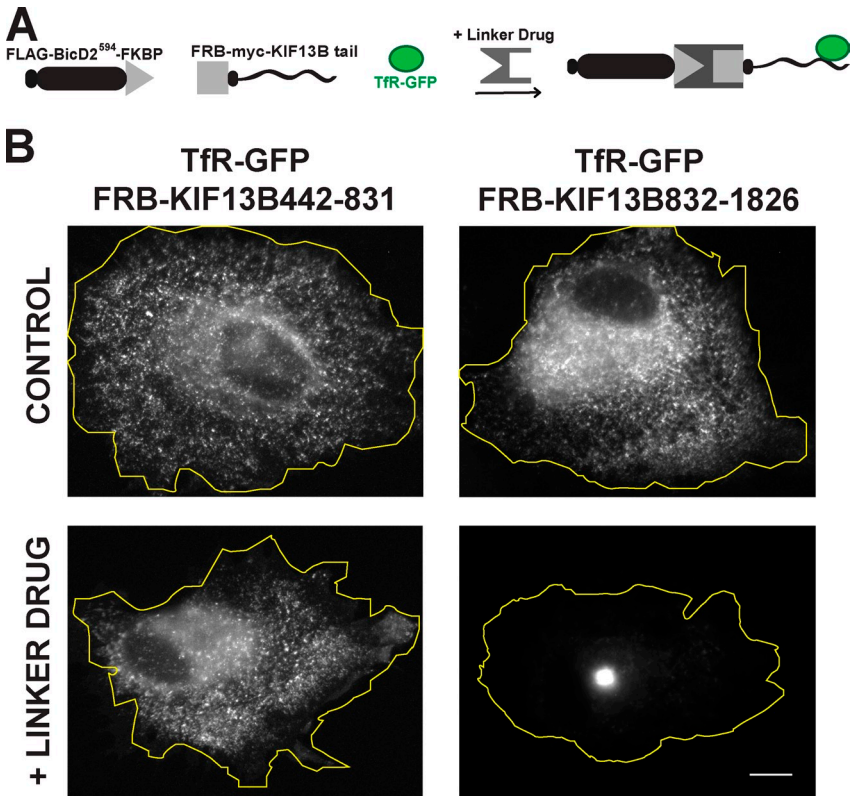

Figure 5. **Identifying the domain of KIF13B that binds to endosomes.** (A) As shown in the schematic, different regions of KIF13B tail tagged with FRB were coexpressed with tdTM-BicD2^{594}-FKBP and TfR-GFP. (B) In control cells, TfR-GFP was distributed throughout the cell. After treatment with the linker drug TfR-GFP, vesicles were moved to the cell center in cells expressing FRB-3myc-KIF13B$^{832–1,826}$ but not FRB-3myc-KIF13B$^{442–831}$. The yellow lines outline the cell boundaries. Bar, 30 µm.

Because binding of a candidate protein causes the vesicles of interest to move to a well-defined end point in the cell, positive interactions result in an unambiguous change in vesicle distribution that is easily interpretable. This makes it possible to evaluate the results by eye, thus allowing rapid screening of multiple combinations of candidate proteins and vesicle populations. Vesicles can be labeled by any convenient method, including the expression of fluorescent proteins, labeling with fluorescent dyes, or immunostaining.

Although the FRB-tagged candidate proteins are overexpressed, we saw little evidence that this led to nonspecific binding. For example, KIF1A and KIF1Bβ never associated with early endosomes and KIF13A and KIF13B never bound late endosomes or lysosomes. Vesicles may change their identity over time, shedding some trafficking proteins and gaining others. Thus, it is possible that an FRB-tagged protein could bind an unlabeled vesicle and induce a change in its localization; if the identity of that vesicle then changed, it could become labeled after it had been misdirected. The mislocalization of some LysoTracker red vesicles that occurred in cells expressing FRB-Rab5 may be an example of this (Fig. S2).

As with any assay, negative results must be interpreted with caution. If the FKBP- and FRB-tagged constructs are not expressed at sufficiently high levels, too few motors will become linked to the vesicles to move them to the intended destination. If a significant fraction of the FKBP-tagged motor becomes linked to FRB-tagged proteins that are not associated with vesicles—either because the expression level of the FRB-tagged protein is too high or because there is a large pool of unbound FRB protein—this could also result in too few vesicle-bound motors to produce effective movements. False negatives could also arise if the FRB-tagged protein cycles off the vesicle membrane quickly or if the FRB epitope becomes inaccessible for linking to FKBP. Despite these potential caveats, the conditions needed to obtain a positive result were not difficult to achieve.

We believe this assay will have broad application for investigating interactions between trafficking proteins and different vesicle populations. Once a set of FRB-tagged constructs has been prepared, they can be screened against a large number of vesicle populations in a remarkably short period of time. This assay may be particularly useful for evaluating the binding of trafficking proteins that are members of large families, such as Rabs, where any given vesicle is likely to bind a limited number of family members. If different family members bind different vesicles in this assay, this establishes that the FRB-tagged proteins are capable of binding and translocating vesicles but do not bind indiscriminately. This assay can also be adapted to explore other aspects of protein–vesicle interactions. By using one GFP-tagged Kinesin-3 to label vesicles, it was possible to establish that a second Kinesin-3 family member also bound to the same vesicle. The assay can also be used to identify the regions of trafficking proteins that mediate vesicle binding.

The role of Kinesin-3 family members in endosomal transport

We show that early and late endosomes bind different members of the Kinesin-3 family with high specificity. KIF13A and KIF13B, two closely related Kinesin-3s, bind early endosomes. KIF1A and KIF1Bβ, two other Kinesin-3s that are also closely related to each other, bind late endosomes and lysosomes. These conclusions are based on consistent results obtained with all

Table 1. **Constructs used in the induced dimerization assay**

Construct	Construct design[a]	Accession number
FRB-KIF1A tail	FRB-3myc-YID-KIF1A$^{391–1,698}$	NM_008440
FRB-KIF1Bβ tail	FRB-3myc-YKGGSGG-KIF1Bβ$^{387–1,770}$	NM_207682
FRB-KIF13A tail	FRB-3myc-YKYSDLELKLRILQSTVPRA-KIF13A$^{361–1,749}$	NM_010617
FRB-KIF13B$^{442–1,826}$ (full length) tail	FRB-3myc-YKGGGSGGGSGGG-KIF13B$^{442–1,826}$	NM_001081177
FRB-KIF13B$^{442–831}$ (N-terminal fragment) tail	FRB-3myc-YKGGGSGGGSGGG-KIF13B$^{442–831}$	NM_001081177
FRB-KIF13B$^{832–1,826}$ (C-terminal fragment) tail	FRB-3myc-YKGGSGGSGG-KIF13B$^{832–1,826}$	NM_001081177
GFP-BicD2-FKBP	GFP-MID-FLAG-SH-BicD2$^{1–594}$-RTVSR-FKBP	AJ250106
tdTM-BicD2-FKBP	tdTM-MID-FLAG-SH-BicD2$^{1–594}$-RTVSR-FKBP	AJ250106
BicD2-FKBP	FLAG-SH-BicD2$^{1–594}$-RTVSR-FKBP	AJ250106
KIF5C-tdTM-FKBP	KIF5C$^{1–559}$-ELGAPRPT-tdTM-FKBP	NM_001107730
FRB-Rab5	FRB-3myc-YKRTGSG-Rab5a	NM_025887
FRB-Rab7	FRB-3myc-YKRTGSG-Rab7	NM_009005

Accession numbers were obtained from GenBank.
[a]Includes epitope tag or label (FLAG, myc, GFP, or tdTM), heterodimerization domain (FRB or FKBP), amino acid linker sequence (using single letter code), and trafficking protein (amino acids included).

seven of the markers we used to label these two endosomal populations (Figs. 3 and S3).

Although the work described here is the first systematic investigation of the interaction of Kinesin-3s with different endosomal populations, our findings are consistent with previous studies that implicated one or another Kinesin-3 in different aspects of endosomal trafficking. Kinesin-73, a *Drosophila* KIF13 homologue, binds Rab5 vesicles in S2 cells (Huckaba et al., 2011), KIF13A plays a role in tubule formation at sorting endosomes (Delevoye et al., 2009), and KIF13B has been implicated in endocytosis in hepatocytes (Kanai et al., 2014). In contrast, KIF1Bβ binds lysosomes in COS7 cells (Matsushita et al., 2004). KIF16B, a Kinesin-3 we did not examine in this study, is also involved in endocytic trafficking (Hoepfner et al., 2005; Blatner et al., 2007). The new assay we developed allowed a systematic approach to this question, which revealed a pattern of Kinesin-3–endosome binding that appears to be consistent over a range of cell types and species but that was not apparent from earlier studies that examined one kinesin and its interaction with a single population of vesicles.

Conclusions

In the experiments presented here, we introduce a new method to identify protein–vesicle interactions in intact cells. Using an inducible dimerization system to link candidate vesicle-binding proteins to activated motors, the association of a candidate protein with a labeled vesicle is transduced into a predictable, highly distinctive change in vesicle localization. This output provides direct experimental evidence of vesicle binding, in contrast to approaches such as two-color colocalization, which are essentially correlative. Using this approach to investigate trafficking proteins that bind early and late endosomes, we show that the assay has a high specificity, a wide range of utility, and a readout that is easily interpretable.

Materials and methods

Constructs

All constructs were cloned into the pCAG expression vector (Niwa et al., 1991). This expression system consists of a cytomegalovirus–immediate early enhancer combined with a chicken β-actin promoter and has been shown to express at steady levels. We generated the following constructs: KIF5C^{559}-tdTM-FKBP (Friedman and Vale, 1999; Jacobson et al., 2006), tdTM-Bicaudal^{594}-FKBP, and FLAG-Bicaudal^{594}-FKBP (Kapitein et al., 2010a). FRB-tagged Rab proteins were prepared by inserting the FRB-3myc sequence at their N termini. FRB-tagged kinesin tails were engineered by removing the N-terminal motor domain and the first coiled-coiled domain, replacing them with an FRB-3myc domain. Details about linkers, accession numbers, and tags for each of these constructs can be found in Table 1. The following cDNAs were used to label specific vesicle populations: mouse Rab5a (GFP-Rab5; GenBank accession number NM_025887) or Rab7 (GFP-Rab7; GenBank accession number NM_009005) tagged with EGFP at their N termini, human TfR tagged with GFP at its C terminus (TfR-GFP; Burack et al., 2000; Silverman et al., 2001), human low density lipoprotein receptor tagged by insertion of EGFP downstream of the signal sequence (GFP–low density lipoprotein receptor; Silverman et al., 2005), and human LAMP1 tagged with a C-terminal GFP (LAMP1-GFP; GenBank accession number J04182).

FRB-tagged constructs included a 3myc epitope tag so that expressing cells could be identified by immunostaining; in preliminary experiments, we established that the great majority of cells that expressed both of the fluorescent constructs also expressed the FRB-tagged protein, based on anti-myc immunostaining.

Cell culture

Rat embryonic fibroblast cells (Heidemann et al., 1999) were grown at 37°C in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum, 4.5 g/liter D-glucose, 548 mg/liter L-glutamine, 110 mg/liter sodium pyruvate, 0.1 g/liter streptomycin, and 100 U/ml penicillin. 1 or 2 d before transfection, cells were trypsinized, and cells were replated on glass coverslips.

Vesicle binding assay

cDNAs encoding each of the components for the vesicle binding assay were expressed by transfection with FuGENE 6 (Promega). Transfected components included the putative vesicle binding protein tagged with FRB, either KIF5C^{559}-tdTM-FKBP, tdTM-Bicaudal^{594}-FKBP, or FLAG-Bicaudal^{594}-FKBP, and in some cases a GFP-tagged protein that served to label the vesicles of interest. After expression for ∼48 h, heterodimerization of the FRB-tagged and FKBP-tagged proteins was induced by treating cells with 100 nM AP21967, a rapamycin analogue (Muthuswamy et al., 1999; Kapitein et al., 2010a). Kinesin tails were designed by removal of the motor and the dimerization domains followed by the addition of an N-terminal FRB-3myc (Table 1). If present, the latter domain could mediate dimerization with endogenous kinesins and hence have a dominant-negative effect (Uchida et al., 2009; Hendricks et al., 2010; Lewis et al., 2011). After 1–3 h with the linker drug, cells were fixed in 4% formaldehyde with 4% sucrose and then mounted in Elvanol (Banker and Goslin, 1998). Control cells were treated identically but not exposed to AP21967. Endogenous lysosomes were labeled by exposing cells for 30 min to 200 nM LysoTracker red (DND-99) before fixation (Molecular Probes). Endogenous early endosomes were labeled by exposing cells for 1 h to 25 µg/ml Alexa

Fluor 555–tagged human transferrin before fixation (Molecular Probes). In some experiments, EEA1-positive endosomes were labeled after fixation by immunostaining with the rabbit monoclonal anti-EEA1 (C45B10; Cell Signaling Technology). Myc-tagged proteins were detected by immunostaining with the mouse monoclonal anti–c-myc 9E10 (M4439; Sigma-Aldrich).

Microscopy

Cells were imaged using an epifluorescence microscope (Axio Observer.Z1; Carl Zeiss) equipped with an LCI Plan Apochromat 40×/1.3 NA or LCI Plan Apochromat 63×/1.4 NA objective. Images of cells were acquired using a camera (AxioCam MRm; Carl Zeiss) and AxioVision software (Carl Zeiss).

Online supplemental material

Fig. S1 shows that linking constitutively active Kinesin-1 to Rab7 endosomes results in their accumulation in the periphery of the cell. Fig. S2 shows that linking FRB-Rab5 to dynein results in the accumulation of early endosomes at the cell center. Fig. S3 shows that different Kinesin-3 family members bind early and late endosomes. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201408056/DC1.

We thank Barbara Smoody for her outstanding technical assistance, Dr. Stefanie Kaech for advice on imaging, and Drs. Fred Robinson, Bruce Schnapp, and Vladimir Gelfand for their comments on the manuscript.

This work was supported by National Institutes of Health grant MH066179. H. Decker was the recipient of the McDonald Fellowship from the Multiple Sclerosis International Federation.

The authors declare no competing financial interests.

Submitted: 12 August 2014
Accepted: 26 December 2014

References

Banker, G., and K. Goslin. 1998. Culturing Nerve Cells. Second edition. MIT Press, Cambridge, MA.666 pp.

Belshaw, P.J., S.N. Ho, G.R. Crabtree, and S.L. Schreiber. 1996. Controlling protein association and subcellular localization with a synthetic ligand that induces heterodimerization of proteins. *Proc. Natl. Acad. Sci. USA*. 93:4604–4607. http://dx.doi.org/10.1073/pnas.93.10.4604

Blatner, N.R., M.I. Wilson, C. Lei, W. Hong, D. Murray, R.L. Williams, and W. Cho. 2007. The structural basis of novel endosome anchoring activity of KIF16B kinesin. *EMBO J.* 26:3709–3719. http://dx.doi.org/10.1038/sj.emboj.7601800

Bucci, C., P. Thomsen, P. Nicoziani, J. McCarthy, and B. van Deurs. 2000. Rab7: a key to lysosome biogenesis. *Mol. Biol. Cell*. 11:467–480. http://dx.doi.org/10.1091/mbc.11.2.467

Burack, M.A., M.A. Silverman, and G. Banker. 2000. The role of selective transport in neuronal protein sorting. *Neuron*. 26:465–472. http://dx.doi.org/10.1016/S0896-6273(00)81178-2

Cai, D., D.P. McEwen, J.R. Martens, E. Meyhofer, and K.J. Verhey. 2009. Single molecule imaging reveals differences in microtubule track selection between Kinesin motors. *PLoS Biol.* 7:e1000216. http://dx.doi.org/10.1371/journal.pbio.1000216

Delevoye, C., I. Hurbain, D. Tenza, J.-B. Sibarita, S. Uzan-Gafsou, H. Ohno, W.J.C. Geerts, A.J. Verkleij, J. Salamero, M.S. Marks, and G. Raposo. 2009. AP-1 and KIF13A coordinate endosomal sorting and positioning during melanosome biogenesis. *J. Cell Biol.* 187:247–264. http://dx.doi.org/10.1083/jcb.200907122

Dienstbier, M., and X. Li. 2009. Bicaudal-D and its role in cargo sorting by microtubule-based motors. *Biochem. Soc. Trans.* 37:1066–1071. http://dx.doi.org/10.1042/BST0371066

Duclos, S., G. Clavarino, G. Rousserie, G. Goyette, J. Boulais, V. Camossetto, E. Gatti, S. LaBoissière, P. Pierre, and M. Desjardins. 2011. The endosomal proteome of macrophage and dendritic cells. *Proteomics*. 11:854–864. http://dx.doi.org/10.1002/pmic.201000577

Franzusoff, A., E. Lauzé, and K.E. Howell. 1992. Immuno-isolation of Sec7p-coated transport vesicles from the yeast secretory pathway. *Nature*. 355:173–175. http://dx.doi.org/10.1038/355173a0

Friedman, D.S., and R.D. Vale. 1999. Single-molecule analysis of kinesin motility reveals regulation by the cargo-binding tail domain. *Nat. Cell Biol.* 1:293–297. http://dx.doi.org/10.1038/13008

Hanada, T., L. Lin, E.V. Tibaldi, E.L. Reinherz, and A.H. Chishti. 2000. GAKIN, a novel kinesin-like protein associates with the human homologue of the *Drosophila* discs large tumor suppressor in T lymphocytes. *J. Biol. Chem.* 275:28774–28784. http://dx.doi.org/10.1074/jbc.M000715200

Heidemann, S.R., S. Kaech, R.E. Buxbaum, and A. Matus. 1999. Direct observations of the mechanical behaviors of the cytoskeleton in living fibroblasts. *J. Cell Biol.* 145:109–122. http://dx.doi.org/10.1083/jcb.145.1.109

Hendricks, A.G., E. Perlson, J.L. Ross, H.W. Schroeder III, M. Tokito, and E.L.F. Holzbaur. 2010. Motor coordination via a tug-of-war mechanism drives bidirectional vesicle transport. *Curr. Biol.* 20:697–702. http://dx.doi.org/10.1016/j.cub.2010.02.058

Hoepfner, S., F. Severin, A. Cabezas, B. Habermann, A. Runge, D. Gillooly, H. Stenmark, and M. Zerial. 2005. Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B. *Cell*. 121:437–450. http://dx.doi.org/10.1016/j.cell.2005.02.017

Huckaba, T.M., A. Gennerich, J.E. Wilhelm, A.H. Chishti, and R.D. Vale. 2011. Kinesin-73 is a processive motor that localizes to Rab5-containing organelles. *J. Biol. Chem.* 286:7457–7467. http://dx.doi.org/10.1074/jbc.M110.167023

Jacobson, C., B. Schnapp, and G.A. Banker. 2006. A change in the selective translocation of the Kinesin-1 motor domain marks the initial specification of the axon. *Neuron*. 49:797–804. http://dx.doi.org/10.1016/j.neuron.2006.02.005

Jenkins, B., H. Decker, M. Bentley, J. Luisi, and G. Banker. 2012. A novel split kinesin assay identifies motor proteins that interact with distinct vesicle populations. *J. Cell Biol.* 198:749–761. http://dx.doi.org/10.1083/jcb.201205070

Kanai, Y., D. Wang, and N. Hirokawa. 2014. KIF13B enhances the endocytosis of LRP1 by recruiting LRP1 to caveolae. *J. Cell Biol.* 204:395–408. http://dx.doi.org/10.1083/jcb.201309066

Kapitein, L.C., M.A. Schlager, M. Kuijpers, P.S. Wulf, M. van Spronsen, F.C. MacKintosh, and C.C. Hoogenraad. 2010a. Mixed microtubules steer dynein-driven cargo transport into dendrites. *Curr. Biol.* 20:290–299. http://dx.doi.org/10.1016/j.cub.2009.12.052

Kapitein, L.C., M.A. Schlager, W.A. van der Zwan, P.S. Wulf, N. Keijzer, and C.C. Hoogenraad. 2010b. Probing intracellular motor protein activity using an inducible cargo trafficking assay. *Biophys. J.* 99:2143–2152. http://dx.doi.org/10.1016/j.bpj.2010.07.055

Kardon, J.R., and R.D. Vale. 2009. Regulators of the cytoplasmic dynein motor. *Nat. Rev. Mol. Cell Biol.* 10:854–865. http://dx.doi.org/10.1038/nrm2804

Lewis, T.L., Jr., T. Mao, and D.B. Arnold. 2011. A role for myosin VI in the localization of axonal proteins. *PLoS Biol.* 9:e1001021. http://dx.doi.org/10.1371/journal.pbio.1001021

Matsushita, M., S. Tanaka, N. Nakamura, H. Inoue, and H. Kanazawa. 2004. A novel kinesin-like protein, KIF1Bbeta3 is involved in the movement of lysosomes to the cell periphery in non-neuronal cells. *Traffic*. 5:140–151. http://dx.doi.org/10.1111/j.1600-0854.2003.00165.x

Muthuswamy, S.K., M. Gilman, and J.S. Brugge. 1999. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. *Mol. Cell. Biol.* 19:6845–6857.

Nakata, T., S. Niwa, Y. Okada, F. Perez, and N. Hirokawa. 2011. Preferential binding of a kinesin-1 motor to GTP-tubulin-rich microtubules underlies polarized vesicle transport. *J. Cell Biol.* 194:245–255. http://dx.doi.org/10.1083/jcb.201104034

Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*. 108:193–199. http://dx.doi.org/10.1016/0378-1119(91)90434-D

Poteryaev, D., S. Datta, K. Ackema, M. Zerial, and A. Spang. 2010. Identification of the switch in early-to-late endosome transition. *Cell*. 141:497–508. http://dx.doi.org/10.1016/j.cell.2010.03.011

Rhee, H.-W., P. Zou, N.D. Udeshi, J.D. Martell, V.K. Mootha, S.A. Carr, and A.Y. Ting. 2013. Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. *Science*. 339:1328–1331. http://dx.doi.org/10.1126/science.1230593

Rink, J., E. Ghigo, Y. Kalaidzidis, and M. Zerial. 2005. Rab conversion as a mechanism of progression from early to late endosomes. *Cell*. 122:735–749. http://dx.doi.org/10.1016/j.cell.2005.06.043

Robinson, M.S., D.A. Sahlender, and S.D. Foster. 2010. Rapid inactivation of proteins by rapamycin-induced rerouting to mitochondria. *Dev. Cell*. 18:324–331. http://dx.doi.org/10.1016/j.devcel.2009.12.015

Sengupta, D., S. Truschel, C. Bachert, and A.D. Linstedt. 2009. Organelle tethering by a homotypic PDZ interaction underlies formation of the Golgi membrane network. *J. Cell Biol.* 186:41–55. http://dx.doi.org/10.1083/jcb.200902110

Silverman, M.A., S. Kaech, M. Jareb, M.A. Burack, L. Vogt, P. Sonderegger, and G. Banker. 2001. Sorting and directed transport of membrane proteins during development of hippocampal neurons in culture. *Proc. Natl. Acad. Sci. USA*. 98:7051–7057. http://dx.doi.org/10.1073/pnas.111146198

Silverman, M.A., R. Peck, G. Glover, C. He, C. Carlin, and G. Banker. 2005. Motifs that mediate dendritic targeting in hippocampal neurons: a comparison with basolateral targeting signals. *Mol. Cell. Neurosci.* 29:173–180. http://dx.doi.org/10.1016/j.mcn.2005.02.008

Spang, A. 2008. Membrane traffic in the secretory pathway: The life cycle of a transport vesicle. *Cell. Mol. Life Sci.* 65:2781–2789. http://dx.doi.org/10.1007/s00018-008-8349-y

Takamori, S., M. Holt, K. Stenius, E.A. Lemke, M. Grønborg, D. Riedel, H. Urlaub, S. Schenck, B. Brügger, P. Ringler, et al. 2006. Molecular anatomy of a trafficking organelle. *Cell*. 127:831–846. http://dx.doi.org/10.1016/j.cell.2006.10.030

Tong, Y., W. Tempel, H. Wang, K. Yamada, L. Shen, G.A. Senisterra, F. MacKenzie, A.H. Chishti, and H.-W. Park. 2010. Phosphorylation-independent dual-site binding of the FHA domain of KIF13 mediates phosphoinositide transport via centaurin α1. *Proc. Natl. Acad. Sci. USA*. 107:20346–20351. http://dx.doi.org/10.1073/pnas.1009008107

Uchida, A., N.H. Alami, and A. Brown. 2009. Tight functional coupling of kinesin-1A and dynein motors in the bidirectional transport of neurofilaments. *Mol. Biol. Cell*. 20:4997–5006. http://dx.doi.org/10.1091/mbc.E09-04-0304

Vale, R.D. 2003. The molecular motor toolbox for intracellular transport. *Cell*. 112:467–480. http://dx.doi.org/10.1016/S0092-8674(03)00111-9

Vale, R.D., T. Funatsu, D.W. Pierce, L. Romberg, Y. Harada, and T. Yanagida. 1996. Direct observation of single kinesin molecules moving along microtubules. *Nature*. 380:451–453. http://dx.doi.org/10.1038/380451a0

van Spronsen, M., M. Mikhaylova, J. Lipka, M.A. Schlager, D.J. van den Heuvel, M. Kuijpers, P.S. Wulf, N. Keijzer, J. Demmers, L.C. Kapitein, et al. 2013. TRAK/Milton motor-adaptor proteins steer mitochondrial trafficking to axons and dendrites. *Neuron*. 77:485–502. http://dx.doi.org/10.1016/j.neuron.2012.11.027

Wickner, W., and R. Schekman. 2008. Membrane fusion. *Nat. Struct. Mol. Biol.* 15:658–664. http://dx.doi.org/10.1038/nsmb.1451

Zhang, L., G.S. Katselis, R.E. Moore, K. Lekpor, R.M. Goto, T.D. Lee, and M.M. Miller. 2011. Proteomic analysis of surface and endosomal membrane proteins from the avian LMH epithelial cell line. *J. Proteome Res.* 10:3973–3982. http://dx.doi.org/10.1021/pr200179r