

# Arf6, Rab11 and transferrin receptor define distinct populations of recycling endosomes

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Recycling endosomes are key platforms for endocytic recycling that return internalized molecules back to the plasma membrane. To determine how recycling endosomes perform their functions, searching for proteins and lipids that specifically localized at recycling endosomes has often been performed by colocalization analyses between candidate molecules and conventional recycling endosome markers. However, it remains unclear whether all the conventional markers have identical localizations. Here we report finding that three well-known recycling endosome markers, i.e., Arf6, Rab11 and transferrin receptor (TfR), have different intracellular localizations in PC12 cells. The results of immunofluorescence analyses showed that the signals of endogenous Arf6, Rab11 and TfR in nerve growth factor-stimulated PC12 cells generally differed, although there was some overlapping. Our findings provide new information about recycling endosome markers, and they highlight the heterogeneity of recycling endosomes.

Endocytic recycling is an intracellular process that returns internalized molecules back to the plasma membrane,<sup>1,2</sup> and it plays crucial roles not only in the reuse of receptor molecules but in remodeling of the protein and lipid composition of the plasma membrane. Thus, endocytic recycling is required for a variety of cellular events, including cell migration, cytokinesis and neurite outgrowth.<sup>3-5</sup> One of the platforms that plays a central role in endocytic recycling is the recycling endosome.<sup>6-8</sup> Recycling endosomes are usually localized in the perinuclear area, and they function as endosomal hubs that connect endocytic and exocytic membrane trafficking by receiving internalized molecules from early endosomes and returning them to the plasma membrane. However, the molecular mechanisms by which they perform their functions are not fully understood.

To identify the molecular mechanisms by which recycling endosomes perform their functions, searching for proteins and lipids that specifically localized at recycling endosomes has often been performed by colocalization analyses between candidate molecules and conventional recycling endosome markers, e.g., the Arf family small GTPase Arf6, the Rab family small GTPase Rab11 and the transferrin receptor (TfR).<sup>2,9,10</sup> Thus, candidate molecules that colocalize with Arf6, Rab11 or TfR have been simply referred to as “recycling endosomal” molecules in the literature. However, it is unclear whether the intracellular localizations of Arf6, Rab11 or TfR are the same or not.

We previously reported finding that Rab35, which regulates endocytic recycling and promotes neurite outgrowth,<sup>11-14</sup> was highly colocalized with Arf6 in the pericentrosomal area of

nerve growth factor (NGF)-stimulated PC12 cells,<sup>15</sup> indicating that Rab35 also localizes at recycling endosomes. However, the clear difference between the distribution of Rab35 signals and Rab11 signals in NGF-stimulated PC12 cells<sup>15</sup> led us to hypothesize that the intracellular localizations of conventional recycling endosomal proteins are not necessarily the same. In the present study, we investigated whether the three well-known recycling endosome marker proteins Arf6, Rab11 and TfR have different intracellular localizations by visualizing endogenous proteins with specific antibodies.

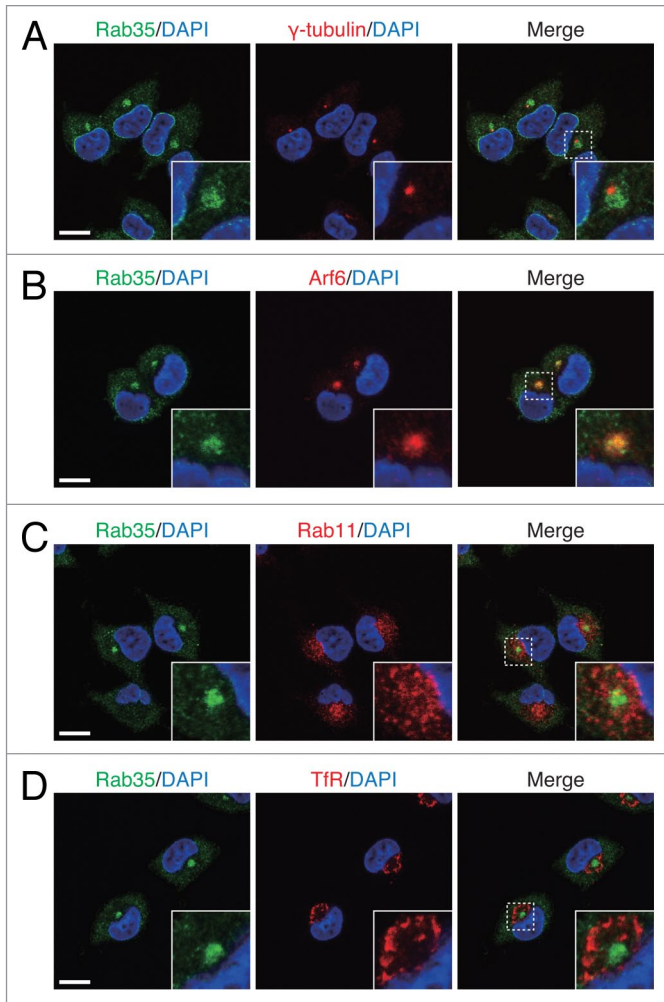
First, we performed colocalization analyses of Rab35 and the three recycling endosome marker proteins in NGF-stimulated PC12 cells. Consistent with the findings in our previous report, the results showed that Rab35 was highly colocalized with Arf6 in the pericentrosomal area (Fig. 1A and B), but that it was only partially colocalized with Rab11 and hardly colocalized with TfR (Fig. 1C and D). We then performed colocalization analyses of the three recycling endosome marker proteins in NGF-stimulated PC12 cells. The results showed generally distinct intracellular localizations of Arf6, Rab11 and TfR, although there was some overlapping, and Arf6, Rab11 and TfR were generally concentrically localized in the perinuclear area, with Arf6 localized closest to the centrosome and TfR farthest from the centrosome (Fig. 2; Fig. S1).

Since TfR continuously recycles between the plasma membrane and recycling endosomes through early endosomes, and newly synthesized TfR passes through the Golgi en route to the plasma membrane, the distinct intracellular localization of TfR

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**Figure 1.** Rab35 highly colocalized with Arf6, but only partially colocalized with Rab11 and hardly colocalized with TfR. After stimulating PC12 cells with NGF for 6 h, the cells were fixed and then stained with anti-Rab35 antibody and DAPI together with anti- $\gamma$ -tubulin antibody (A), anti-Arf6 antibody (B), anti-Rab11 antibody (C) or anti-TfR antibody (D). The insets show magnified views of the boxed areas. Scale bars, 10  $\mu$ m.

colocalized with EEA-1 (an early endosome marker) and GM130 (a Golgi marker), the majority of TfR-positive compartments should be regarded as recycling endosomes (Fig. S2). We therefore concluded that the distinct intracellular localizations of Arf6, Rab11 and TfR reflect the heterogeneity of recycling endosomes.

Interestingly, the immunofluorescence signals of Arf6, but not of Rab11 or TfR, were changed in response to NGF stimulation. In the absence of NGF stimulation, only faint Arf6 signals were observed in the perinuclear area, whereas the Arf6 signals increased in response to NGF stimulation (Fig. 3A). By contrast, the Rab11 signals and TfR signals did not undergo any marked changes during NGF stimulation. Because the level of Arf6 expression was unchanged by NGF stimulation (Fig. 3B), the formation of Arf6-positive recycling endosomes is likely to be promoted by NGF signals. We speculate that Arf6-positive recycling endosomes have a specific role in endocytic recycling that facilitates neurite outgrowth of PC12 cells.

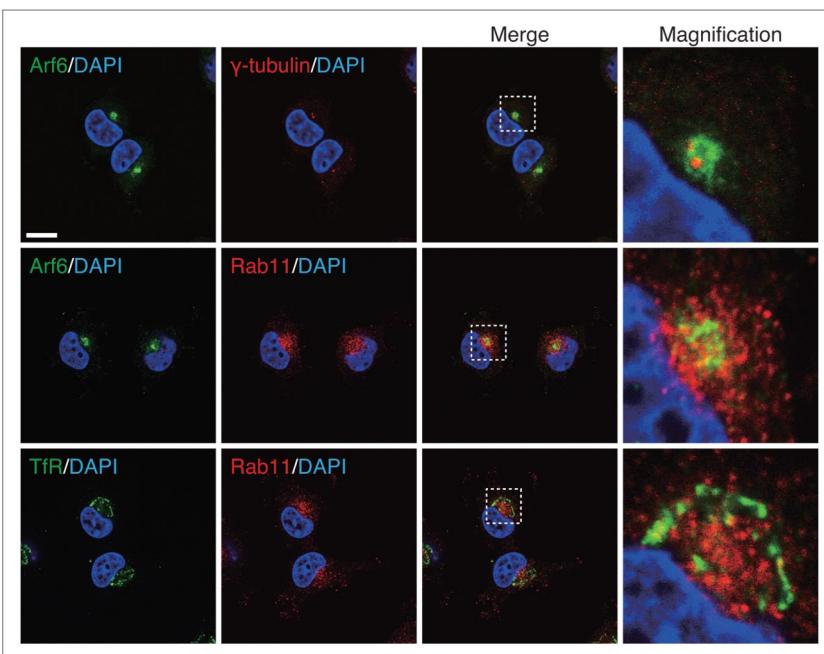
Our findings provide new information about recycling endosome markers, i.e., the localizations of Arf6, Rab11 and TfR only partially overlap in PC12 cells. Thus, a combination of recycling endosome markers should be used to determine whether a candidate molecule localizes to recycling endosomes. Our findings also highlight the heterogeneity of recycling endosomes in PC12 cells (Fig. 4). Recycling endosomes have been reported to regulate not only endocytic recycling but also exocytic and retrograde membrane trafficking,<sup>16,17</sup> indicating that recycling endosomes have multiple sorting functions. Therefore, the different intracellular localizations of the recycling endosome marker proteins may

and Rab11 (or Arf6) can be explained by the majority of TfR proteins being localized on early endosomes and/or the Golgi. However, because only small amounts of TfR were found to be

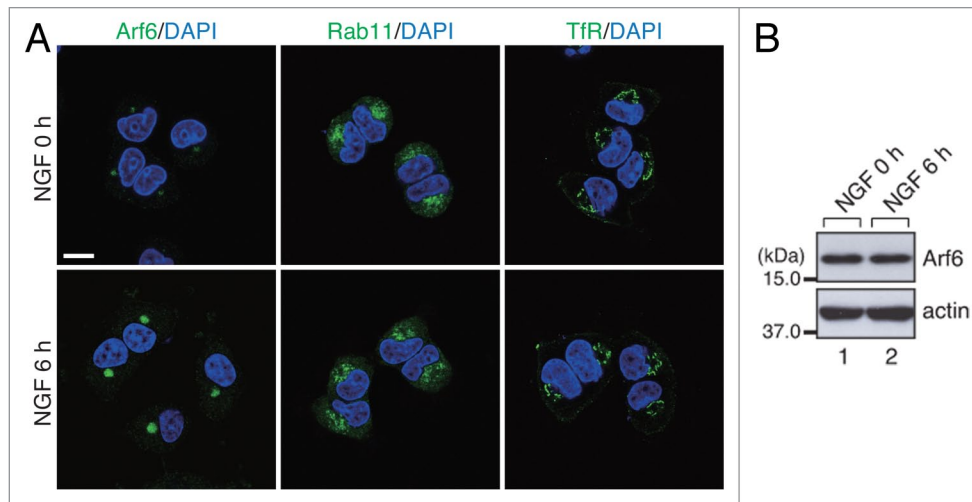
reflect the presence of functionally distinct populations of recycling endosomes. In the future, endogenous recycling endosomal proteins should be visualized in cell types other than PC12 cells to determine whether recycling endosome heterogeneity exists in many cell types. In any event, extensive research will be necessary to understand the physiological significance of the heterogeneity of recycling endosomes.

## Materials and Methods

**Antibodies.** Anti-Rab35 antibody was prepared as described previously.<sup>15,18</sup> Anti- $\gamma$ -tubulin mouse



**Figure 2.** Arf6, Rab11 and TfR have distinct intracellular localizations. After stimulating PC12 cells with NGF for 6 h, the cells were fixed and stained with antibodies against the recycling endosome marker proteins indicated and with DAPI. The panels on the right are magnified views of the boxed areas in the panels on the left. Scale bars, 10  $\mu$ m.



**Figure 3.** Accumulation of Arf6 signals in the pericentrosomal area in response to NGF stimulation. **(A)** After NGF stimulation for 0 h (no NGF-treatment) and 6 h, PC12 cells were fixed and stained with anti-Arf6 antibody, anti-Rab11 antibody and anti-TfR antibody together with DAPI. Scale bar, 10  $\mu$ m. **(B)** Unaltered expression of Arf6 in PC12 cells during NGF stimulation. After NGF stimulation for 0 h and 6 h, cell lysates of PC12 cells were immunoblotted with anti-Arf6 antibody and anti-actin antibody.

monoclonal antibody (Sigma-Aldrich Corp), anti-Arf6 mouse monoclonal antibody (Santa Cruz Biotechnology, Inc), anti-Rab11 rabbit polyclonal antibody, anti-TfR mouse monoclonal antibody (Invitrogen Corp), anti-EEA-1 mouse monoclonal antibody and anti-GM130 mouse monoclonal antibody (BD Biosciences) were obtained commercially. The immunostaining in Figs. S1 and S2 was performed with a fluorophore-conjugated anti-TfR antibody that was generated by using the Zenon Antibody Labeling Kit (Invitrogen Corp). Using this antibody slightly enhanced the TfR signals at the plasma membrane (Figs. S1 and S2).

**Cell culture, immunoblotting and immunofluorescence analyses.** All of the procedures used to perform cell culture, immunoblotting and the immunofluorescence analyses have been described elsewhere.<sup>15,18</sup>

#### Disclosure of Potential Conflicts of Interest

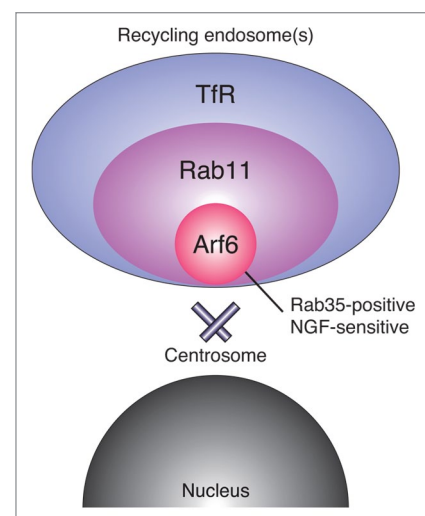
No potential conflicts of interest were disclosed.

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**Figure 4.** A model of the intracellular localizations of recycling endosomal proteins in NGF-stimulated PC12 cells. Arf6, Rab11 and TfR are localized in the perinuclear area, with Arf6 localized closest to the centrosome and TfR farthest from the centrosome, suggesting heterogeneity of the recycling endosomes.

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