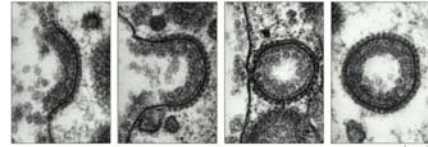


Rab effectors regulate organization of endosomes

Presented by
Joanna Manoranjan
March 10, 2005

Divalent Rab Effectors Regulate the Sub-Compartmental Organization of Early Endosomes

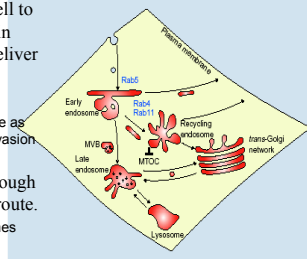
Stefano De Renzi, Birte Sönnichsen and Marino Zerial
Nature Cell Biology, Vol 4, Feb. 2002



Alberts B. Molecular Biology of the Cell, 4th ed. 2002

Endocytosis

- Endocytosis allows the cell to take up macromolecules in **Early Endosomes** and deliver them to lysosomes for digestion or degradation.
 - Important for nutrient intake as well as defense against invasion.
- Involved in **recycling** through either a "fast" or "slow" route.
 - Fast route**: early endosomes return cargo directly to the plasma membrane
 - Slow route**: cargo transported through perinuclear recycling endosomes



Regulation of Endocytosis: Organization and sorting of Early Endosomes

Questions addressed here include:

- How is Endocytosis regulated?
- More specifically, how do Rab proteins and their Effectors regulate endosome organization and sorting?
- How is the Rab5 dependent early endosome sorted into sequential Rab 4 domains?

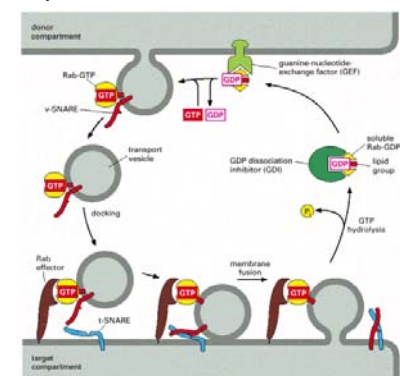
Rab Proteins

- Rab proteins are GTPases (GTP binding proteins)

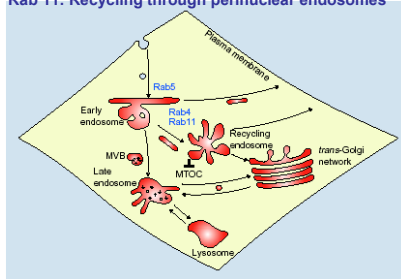


- GAP: GTPase activating protein; induces hydrolyzation of GTP to GDP
- GEF: Guanine nucleotide exchange factor; allows for release of GDP and replacement of GTP

Proposed Role of Rab Proteins and Effectors



- Rab 5 : defines site of early endosome entry
- Rab4: Involved in early recycling pathway/sorting
- Rab 11: Recycling through perinuclear endosomes



Proposed Interactions between Rab5 & its effectors:

Rabaptin-5-Rabex 5 complex →
 activates Rab5 → binds to hVPS34 →
 generates PI(3)P → localizes
 EEA1 & Rabenosyn 5, both
 Rab5 Effectors

- Interested in seeing how Rab5 and Rab4 domains interact
- Purpose here was to identify proteins that interact with both Rab5 and Rab4.
- The method involved two steps of affinity chromatography:



1. Purification of Rab5 interacting proteins using GST-Rab5 loaded with GTPs and incubating with bovine brain cytosol
2. Elution of bound proteins was applied to a second column with GST-Rab4 in the presence of GTPs.

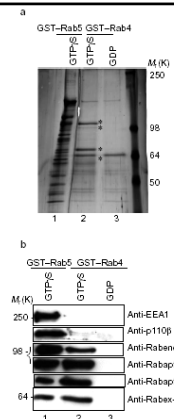
1A. Eluted proteins run on SDS-Page gel with silver staining

- ◆ Lane 1: the proteins found to bind with Rab5
- ◆ Lane 2: the proteins that can also bind to Rab4

- Control: GDP instead of GTP;
Rab proteins remain inactive and no binding occurs.

Four bands found in the Rab4 column:
Rabenosyn5, Rabaptin5, Rabaptin5B, and
Rabex5 (analyzed by Mass Spec)

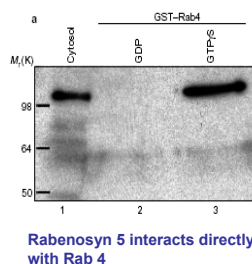
- Rabenosyn5 interaction with Rab4 previously unknown
- Suggests more functions in early endosomes for Rabenosyn5



Does Rabenosyn5 interact directly with Rab4 without the presence of Rab5?

Used affinity chromatography again with GST-Rab4 & incubated with bovine brain cytosol either in the presence of GTP or GDP.

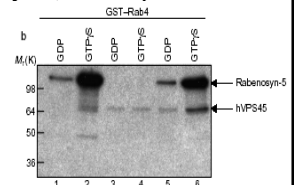
- Bound proteins were eluted; separated by SDS-PAGE
- Analyzed by Western Blot using antibodies against Rabenosyn5



- hVps45 is a Sec1 like protein involved in assembly of endosomal v- and t-snares

- **Fig.2B.** GST-Rab4-GTP or -GDP is incubated with Rabenosyn5 &, or, hVps45 cDNA being transcribed *in vitro*; labelled with ³⁵S-methionine.

Result: hVPS45 needs Rabensyn5 in order to bind to Rab4



2C. Testing Interaction Between Rabenosyn5 and Rab11
(incubation with in vitro translated Rabenosyn5)

No interaction detected

2D. Binding Affinity of Rabenosyn5 to Rab4 and Rab5

Rabenosyn5 shows similar binding affinity for both Rab proteins

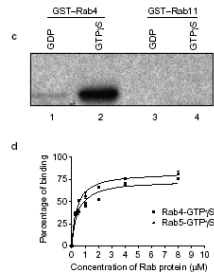
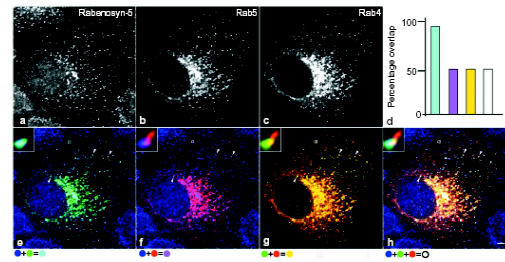


Figure 3. Confocal immunofluorescence analysis of Rab5-, Rab4- and Rabenosyn-5-labelled endosomes



90% of Rabenosyn5 and Rab5 co-localize, while there is about 55% colocalization of other proteins, including all three together.

Figure 4 Rabenosyn-5 binds simultaneously to Rab5 and Rab4

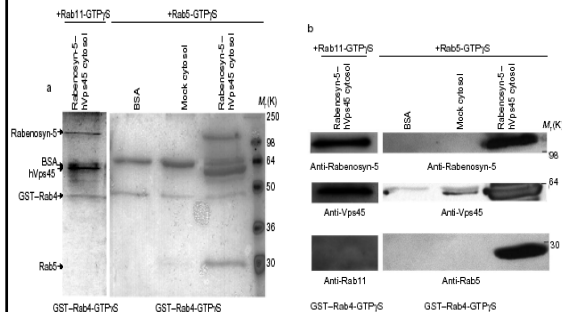
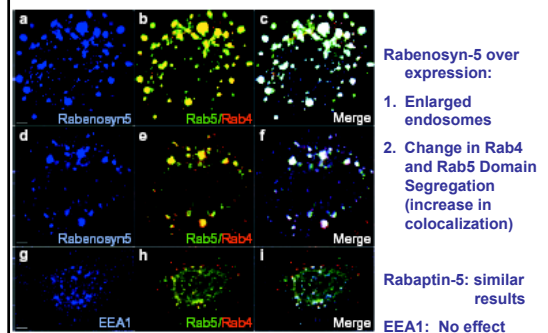


Figure 5. Over expression of Rabenosyn-5, Rabaptin-5, and EEA1

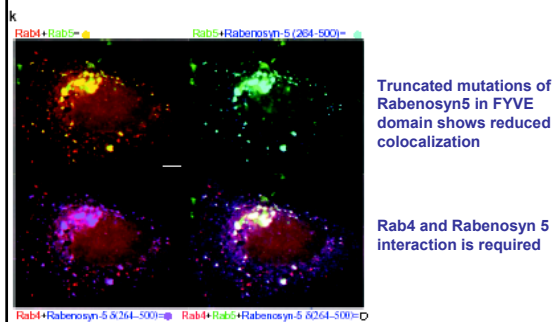


Rabenosyn-5 over expression:

1. Enlarged endosomes
2. Change in Rab4 and Rab5 Domain Segregation (increase in colocalization)

Rabaptin-5: similar results
EEA1: No effect

Figure 5K. Is Rabenosyn5-Rab4 interaction required for the over expression "phenotype"?



Truncated mutations of Rabenosyn5 in FYVE domain shows reduced colocalization

Rab4 and Rabenosyn 5 interaction is required

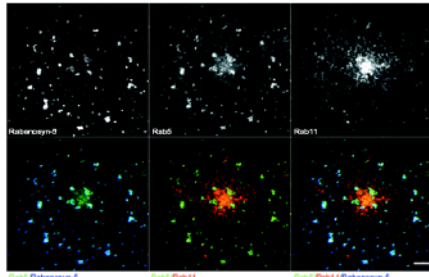
Various Truncation Mutants of Rabenosyn5



Figure 5J

Mutant with amino acid residue 264-500 is required for binding to Rab 4

Figure 6: Effects of Rabenosyn-5 over expression on Rab 11-positive membranes



Rab 11 positive membranes are unaffected by Rabenosyn5 over expression

Summary of the effects of Rabenosyn5 over expression

Table 1

Table 1 Quantification of Rab5, Rab4 and Rab11 overlap on the same endosomes.

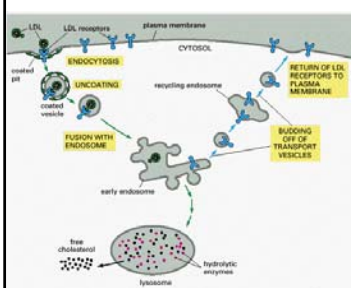
	Rab5 + Rab4	Rab4 + Rab11	Rab5 + Rab4 + Rab11
Control	52 ± 4	50 ± 1	23 ± 2
+Rabenosyn5	90 ± 6	17 ± 3	ND
+Rabenosyn5 (6264-500)	58 ± 5	ND	ND
+hPS45	50 ± 3	ND	ND
+Rabenosyn5 +hPS45	92 ± 4	ND	ND
+Rabaptin5	80 ± 3	ND	ND
+EEA1	60 ± 5	ND	ND

Fluorescence analysis: plasmids encoding the proteins indicated in the first column were microinjected in the nucleus of A431 cells. Numbers represent the percentage of endosomes labelled for the indicated combination of Rab proteins. ND, not determined.

•The number of Rab4-Rab5 positive domains increases

•The number of Rab4-Rab11 domains decreases

Do changes in Rab4-Rab5 domains alter the recycling of Endocytic markers?

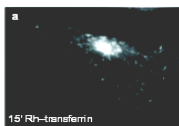


-Decided to look at Transferrin
-a soluble protein that carries iron to the blood

-Good candidate for observing recycling of receptors

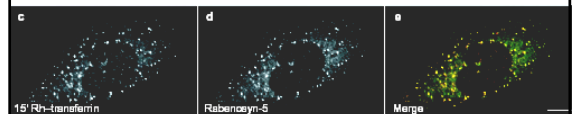
Analysis of Transferrin Transport

Figure 7 A, C-E



Control: Untransfected CHO cells show localization to peripheral early endosomes & perinuclear recycling endosomes

With Rabenosyn 5 over expression, transferrin is transported less efficiently to the perinuclear recycling endosomes



Over expression of Rabenosyn5 promotes the fast recycling pathway directly back to the plasma membrane while slowing the transport to perinuclear recycling endosomes.

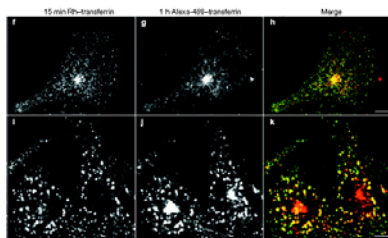
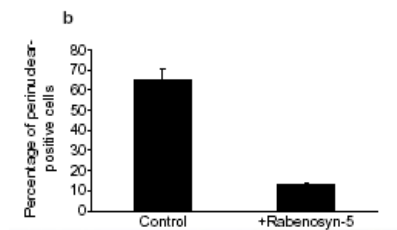
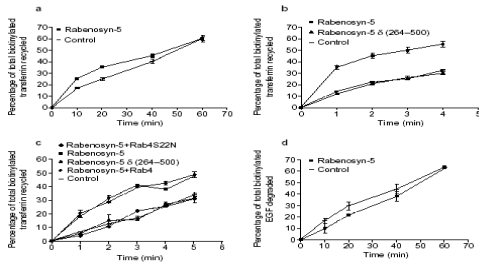


Fig.7 f-k

Cells over expressing Rabenosyn 5 have greatly reduced perinuclear positive cells.



Kinetics of transferrin recycling & EGF degradation



Summary of Results

- The sequential transport of cargo implies coordination with neighboring Rab proteins
- Rabenosyn 5 appears to work with Rabaptin 5 to link the Rab 5 and Rab4 domains.
- Some Rab4 effectors could regulate Rab11 domains.
- Rab5 and Rab4 are in dynamic equilibrium between fission and fusion; Rabenosyn 5 and Rabaptin may act as stabilizers between the two domains to counteract fission.

Proposed Model for Divalent Function of Rabenosyn with Rab4 and Rab5

