Analysis of the role of Rab4 and Rab11 in endosomal carrier formation and transferrin recycling

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Summary

In all eukaryotic cells, membrane transport is essential to distribute proteins from the endoplasmic reticulum to their respective destination, i.e. the Golgi, the endosomal/lysosomal system and the cell exterior. A multitude of proteins is involved in membrane transport, functioning as building blocks for vesicular coats, as regulators of transport, and as targeting and fusion factors. An important set of regulators are the Rab GTPases, which are known to be involved in multiple steps of membrane traffic. However, in many cases, the molecular mechanism of individual transport steps is not yet fully understood. We are interested in the molecular mechanism of two of these Rabs, Rab4 and Rab11, and their function in the late secretory/recycling system.

In order to study the late secretory/recycling system we make use of an *in vitro* permeabilized-cell assay and a novel recycling assay. The *in vitro* permeabilized-cell assay reconstitutes the specific step of carrier formation at endosomes. The function of proteins can be analyzed by modifying the cytosol used in this assay. We have adapted the assay to using cytosol from cultured cells instead of calf brain cytosol. The advantage of cultured cell cytosol is the possibility to modify the composition of the cytosol already *in vivo* by overexpression of wild-type and mutant proteins or by gene silencing. We have also established a novel *in vivo* recycling assay, which makes use of automated immunofluorescence imaging and image analysis software to analyze overall recycling. In this assay, the fluorescence intensity of a reporter cargo can be measured in distinct organelles of thousands of cells at different time points.

For this purpose stable HeLa cell lines expressing dominant negative and constitutively active mutants of Rab4 and Rab11 under an inducible promoter, as well as RNAi knockdown cell lines were created. These cells were then used in the fluorescent recycling assay, and the cytosol of these cells in the *in vitro* assay. Rab4 and Rab11 were both found to be involved in the formation of transport carriers at endosomes containing the transferrin receptor and the EGF receptor. However, *in vivo* the activity of the two Rabs was not essential for overall transferrin recycling. These results suggest that the transferrin receptor is transported in Rab4 and Rab11 dependent carriers, but it is not exclusively dependent on being transported in either of them, and can be efficiently recycled through more than one pathway. Compensatory mechanisms appear to be involved. Future work will investigate the impact on transferrin recycling, when Rab4 and Rab11 are simultaneously impaired.

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Abbreviations

ACAP1	Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 1
ADP	Adenosine diphosphate
ANK	Ankyrin
ANTH	AP180 N-terminal homology
AP	Adaptor protein
Arf	ADP-ribosylation factor
ARH	Autosomal recessive hypercholesterolemia
ASAP1	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1
ATP	Adenosine-5'-triphosphate
BAR	Bin-Amphiphysin-Rvs
BHK	Baby hamster kidney
BiP	Binding protein
BSA	Bovine serum albumin
CDC42	Cell division control protein 42 homolog
CHO	Chinese hamster ovary
CLASP	Clathrin-associated sorting protein
COPI/II	Coat protein I/II
CYFIP	Cytoplasmic FMR1-interacting protein
DAB2	Disabled 2
D-AKAP1	Dual-specific A-kinase-anchoring protein 2
DENN	Differentially expressed in normal and neoplastic cells
Dox	Doxycycline
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosome antigen 1
EGF(R)	Epidermal growth factor (receptor)
EHD	Eps15 homology domain-containing
ENTH	Epsin N-terminal homology
Eps15	EGFR pathway substrate 15
ER	Endoplasmic Reticulum
ERAD	ER associated degradation
ERGIC	ER to Golgi intermediate compartment
ESCRT	Endosomal sorting complex required for transport

FAPP2	Four-phosphate adaptor protein 2
FCH	Fes/CIP4 homology
FCHo	FCH domain only protein
FCS	Fetal calf serum
FGD1	FYVE, RhoGEF and PH domain-containing protein 1
FGF(R)	Fibroblast growth factor (receptor)
FIP	Family interacting protein
FYVE	Fab1, YOTB, Vac1, and EEA1
GAG	Glycosaminoglycan
GAK	Cyclin G-associated kinase
GAP	GTPase activating protein
GAPVD1	GTPase activating protein and VPS9 domain-containing protein 1
GBF1	Golgi-specific brefeldin A-resistant guanine nucleotide exchange factor 1
GDF	GDI dissociation factor
GDI	Rab-GDP dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GGA	Golgi-localized, gamma adaptin ear-containing, Arf-binding protein
GLUT	Glucose transporter
GOLPH3	Golgi phosphoprotein 3
GPI	Glycosylphosphatidylinositol
GRASP-1	GRIP (glutamate receptor interacting protein)-associated protein-1
GTP	Guanosine-5'-triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOPS	Homotypic fusion and vacuole protein sorting
HRB	Arf-GAP domain and FG repeats-containing protein 1
HRP	Horseradish peroxidase
HSC70	Heat shock cognate 70
LDL(R)	Low-density lipoprotein (receptor)
LIMK1	LIM domain kinase 1
MDCK	Madin-Darby canine kidney
MES	2-(N-morpholino)ethanesulfonic acid
MHC	Major histocompatibility complex
MPR	Mannose-6-phosphate receptor
MVB	Multivesicular body

NF-κB	Nuclear factor-κB
NSF	N-ethylmaleimide-sensitive factor
N-WASP	Neural Wiskott-Aldrich syndrome protein
PBS	Phosphate buffered saline
PDI	Protein disulphide isomerase
PFA	Paraformaldehyde
PH	Pleckstrin homology domain
PIC	Protease inhibitor cocktail
PMSF	Phenylmethanesulfonyl fluoride
PtdIns(3)P	Phosphatidylinsoitol (3)-phosphate
PtdIns(4)P	Phosphatidylinositol (4)-phosphate
PtdIns(4,5)P_2	Phosphatidylinositol (4,5)-bisphosphate
Rac1	Ras-related C3 botulinum toxin substrate 1
RCP	Rab coupling protein
REP	Rab escort protein
REP15	Rab effector protein 15
RGS	Regulator of G-protein signaling
RME	Receptor mediated endocytosis
RNAi	RNA interference
SDS	Sodium dodecyl sulfate
SH3	Scr homology 3 domain
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor
SRP	Signal recognition particle
Tf(R)	Transferrin (receptor)
TGF-β	Transforming growth factor-β
TGN	*trans*-Golgi network
TRAPP	Transport protein particle
UPR	Unfolded protein response
VPS45	Vacuolar protein sorting-associated protein 45

Introduction

Eukaryotic cells are divided into membrane-enclosed compartments, so-called organelles, which perform a variety of different specific functions. For example the genetic information is stored in the nucleus, while much of the cell's energy production takes place in mitochondria. Transport of proteins to and from the cell surface occurs via several diverse organelles of the secretory system, and proceeds in a highly efficient and specific manner. Although a lot is known about the processes in the secretory system, there is still a considerable amount of mechanistic understanding lacking, in this complex and elaborate system.

The Secretory System

The secretory system of eukaryotic cells is composed of membrane enclosed compartments through which proteins are transported out of the cell, taken up, recycled, or degraded. The compartments comprising the secretory system are the ER (endoplasmic reticulum), the ERGIC (ER-to-Golgi intermediate compartment), the Golgi apparatus, the endosomes and lysosomes. Organelle identity is defined by their protein and lipid composition, and, to a degree, by their localization within the cell. Transport of proteins between these organelles is mediated by membrane enclosed structures like vesicles or tubules, or by fusion, or maturation of organelles. A schematic representation of these intracellular transport pathways can be found in Figure 1.

In mammalian cells, cytosolic, nuclear, most mitochondrial and peroxisomal proteins are synthesized in the cytosol by free ribosomes. Proteins of the ER, Golgi-apparatus, endosomal-lysosomal system, and the plasma membrane as well as secreted proteins are synthesized at the ER and are translocated into the ER membrane or lumen. From the ER these cargo proteins are transported to the Golgi via the ERGIC, and then move through the Golgi cisternae to the TGN (*trans*-Golgi network). At the TGN the cargo proteins are sorted towards the plasma membrane for secretion in a constitutive or regulated manner, or towards the endosomes and lysosomes.

To ensure specific and efficient transport between these organelles, a large set of proteins is involved, functioning as building blocks for transport intermediates (e.g. vesicles), linkers to motor proteins for directed transport, tethering and fusion factors, and regulatory factors. There is also a retrograde transport system, for example to return organelle specific resident proteins that are constantly moved along with secretory cargo, to their resident organelle.

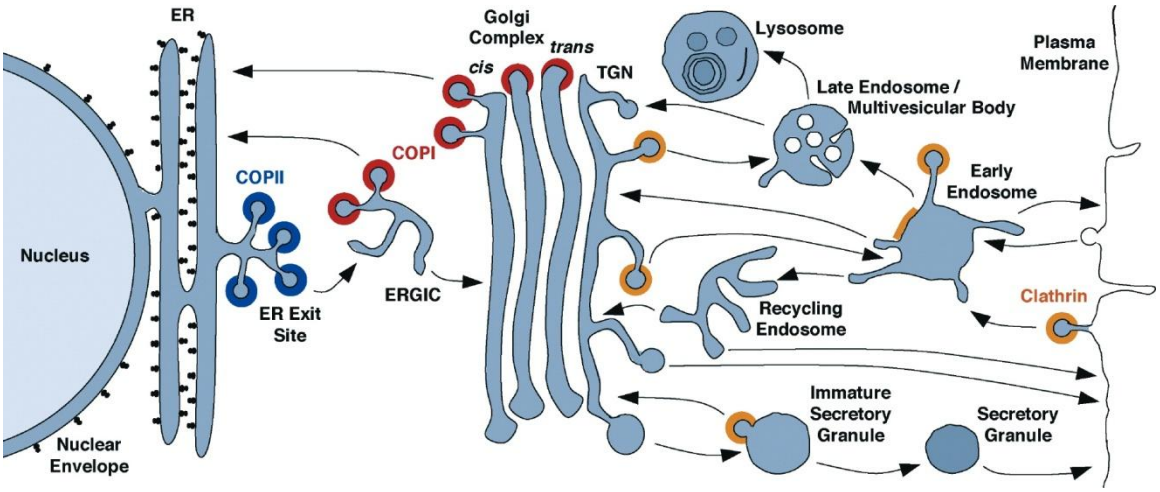

Figure 1. Intracellular transport pathways.

Organelles of the secretory system and the transport routes that exist between them (indicated by arrows) are shown. The colors indicate the known or presumed locations of COPII (blue), COPI (red), and clathrin (orange). Additional coats or coat-like complexes exist as well, but are not represented in this figure. (Reproduced from Bonifacino and Glick, 2004).

The Endoplasmic Reticulum

The ER is a large organelle that consists of an extensive network of membrane tubules and cisternae that are continuous with the nuclear envelope. It is divided into rough ER and smooth ER, depending on the presence or absence of ribosomes. Functions of the ER include protein folding, assembly, quality control and degradation, lipid metabolism, detoxification, and regulation of the calcium household (Lippincott-Schwartz et al., 2000).

To enter the ER, proteins are mostly co-translationally translocated across its membrane. They contain signal sequences that are recognized by the signal recognition particle (SRP) as they emerge from the ribosome. Upon SRP binding translation is halted and the ribosome-nascent chain-SRP complex is targeted to the Sec61 translocon via the interaction of SRP with the SRP receptor (Gilmore et al., 1982; Walter et al., 1982). Upon docking to the membrane, SRP is released and translation is continued, whereby the emerging protein is synthesized through the Sec61 translocon into the ER lumen. Integral membrane proteins are inserted into the membrane by leaving the translocon laterally, the preferred orientation of membrane insertion is determined by the properties of the peptide chain (Goder and Spiess, 2001).

In the ER, proteins are folded and modified. Modifications include disulphide-bond formation, N-linked glycosylation and glycosylphosphatidylinositol (GPI)-anchor attachment. Folding is aided by chaperones that also function as a quality control system which senses incompletely folded proteins to make sure that these proteins do not leave the ER. These chaperones include BiP (binding

protein), calnexin, calreticulin, PDI (protein disulphide isomerase) and others (Ellgaard and Helenius, 2003). Incompletely folded proteins are bound by one of these chaperones and therefore cannot leave the ER. Proteins that are not able to fold are retrotranslocated to the cytoplasm by the ER-associated degradation (ERAD) machinery and are subsequently degraded by the proteasome. If the load of incorrectly folded proteins gets too high, thus leading to ER stress, the unfolded protein response (UPR) is triggered, including down-regulation of general protein synthesis and up-regulation of the synthesis of ER chaperones and ERAD components (Ron and Walter, 2007; Yoshida, 2007). Correctly folded proteins are sorted to, and enriched at ER exit sites, special areas of the ER membrane where COPII vesicles are formed, and are then taken up into COPII vesicles to be transported to the ERGIC (Balch et al., 1994; Barlowe et al., 1994).

The ER-Golgi Intermediate Compartment

In mammalian cells, cargo molecules packaged into COPII vesicles at the ER are transported to the ERGIC. The ERGIC consists of vesicular-tubular membrane clusters and is characterized by the presence of the marker protein ERGIC-53 (Hauri et al., 2000). This organelle serves as a sorting station for anterograde cargo, which is transported onwards to the Golgi, and retrograde transport of cargo back to the Golgi (Ben-Tekaya et al., 2005). ER resident proteins containing an ER retention signal, KDEL for luminal proteins and KKXX or KXKXX (X stands for any amino acid) for membrane proteins, at their C-terminus interact with COPI coat components and are packaged into COPI vesicles for retrograde transport to the ER (Pelham, 1994). Anterograde cargo proteins are packaged into not yet fully characterized anterograde carriers and transported in a microtubule-dependent way to the Golgi (Ben-Tekaya et al., 2005).

The Golgi Complex

The Golgi complex consists of five to eight membrane enclosed cisternae. Typically these cisternae are stacked together, however they can also be dispersed throughout the cytosol, for example in *Saccharomyces cerevisiae* (Preuss et al., 1992). Mammalian cells contain about 40–100 individual Golgi stacks that are interconnected by membrane tubules and can be found in the perinuclear region (Duran et al., 2008). The Golgi can be divided into the cis-Golgi, medial-Golgi and trans-Golgi regions and the TGN, based on the morphology and their enzyme composition. Secretory cargo from the ER/ERGIC enters the Golgi at the cis-face, it is then transported through the Golgi where it is modified and exits the Golgi at the trans-face. Modification is executed by Golgi resident enzymes and includes O-linked glycosylation and processing of N-linked glycans added in the ER.

Glycosaminoglycans (GAGs) are also synthesized on specific proteins in the Golgi to form proteoglycans, which are mostly part of the extracellular matrix. Sulphotransferases add sulphates to GAGs and tyrosine residues of specific proteins. Another important modification is the labeling of lysosomal proteins with mannose-6-phosphate so that they can be recognized by the mannose-6-phosphate receptors (MPRs) for transport to lysosomes.

Intra-Golgi anterograde transport is mediated by cisternal maturation. The cis-face of the Golgi is continuously formed by incoming anterograde transport carriers. The formed cisternae then mature until they reach the trans-face of the Golgi where they are consumed by forming transport carriers. To keep Golgi resident proteins in their correct cisternae, COPI vesicle mediate intra-Golgi retrograde transport (Glick and Malhotra, 1998; Losev et al., 2006; Matsuura-Tokita et al., 2006). Important evidence for cisternal maturation also comes from the fact that large procollagen fibers do not leave the lumen of the cisternae as they migrate through the Golgi (Bonfanti et al., 1998).

Transport from the Golgi back to the ER occurs in a similar way as ERGIC-to-ER transport. The same retention signals (KDEL, KKXX or KXKXX, X is any amino acid) are used and transport is mediated by COPI vesicles. Anterograde cargo is sorted at the trans-most face of the Golgi, the TGN. The destinations of cargo molecules originating at the TGN include the plasma membrane (apical and basolateral in polarized cells), different parts of the endosomal/lysosomal system, and, in endocrine cells, secretory granules (Anitei and Hoflack, 2011). The transport carriers used in TGN exit are pleiomorphic and tubular-vesicular in shape (Polishchuk et al., 2003).

The Endosomal-Lysosomal System

Endosomes are organelles of the late secretory pathway. They are a major sorting station between other endosomes, the plasma membrane and the TGN, receiving and delivering cargo from and to these organelles. For example, the asialoglycoprotein receptor H1 and the transferrin receptor (TfR) are transported from the TGN to endosomes (Futter et al., 1995; Leitinger et al., 1995). From endosomes, cargo proteins are transported back to the TGN (Diaz and Pfeffer, 1998; Ghosh et al., 1998), to the plasma membrane or to the lysosomes (Maxfield and McGraw, 2004).

Endosomes can be divided into three subpopulations, early sorting endosomes, perinuclear recycling endosomes, and late endosomes. Cargo molecules which are internalized from the plasma membrane are first transported to early sorting endosomes. From there, cargo molecules can either be recycled back to the plasma membrane, move further to recycling endosomes, or to late endosomes and subsequently the lysosomes. At the recycling endosomes there is again recycling to

the plasma membrane or transport to the TGN (Bonifacino and Rojas, 2006; Grant and Donaldson, 2009). Recycling from sorting endosomes directly to the plasma membrane is termed fast recycling, while the route via recycling endosomes is termed slow recycling.

Transport from the plasma membrane to the early sorting endosomes is mediated by many different carriers like clathrin coated vesicles, caveolae, and several clathrin- and caveolin-independent vesicles and tubular intermediates (Mayor and Pagano, 2007). Sorting endosomes only accept incoming cargo for a few minutes before they translocate along microtubules, become more acidic and acquire acid hydrolases, and mature into late endosomes (Maxfield and McGraw, 2004). In the process they become multivesicular bodies (MVBs) by the sequential action of the ESCRT (endosomal sorting complex required for transport) machinery which mediates the formation of intralumenal vesicles (Henne et al., 2011). MVBs then become lysosomes by maturation and fusion with preexisting lysosomes, and cargo proteins and intralumenal vesicles are subsequently degraded (Saftig and Klumperman, 2009). Recycling endosomes are thought to be formed by tubules that extend from early sorting endosomes and separate from the main body of sorting endosomes which mature into late endosomes (Grant and Donaldson, 2009; Maxfield and McGraw, 2004). Although many players have been identified, the exact mechanism of endosome to plasma membrane transport is still not entirely resolved. More than one type of carrier may exist which, for example, distinguish between cargoes that were endocytosed in a clathrin dependent or independent way (Grant and Donaldson, 2009). However, clathrin coated vesicles are known to play a part in at least the early endosome to plasma membrane route (van Dam and Stoorvogel, 2002).

Vesicular Transport

Vesicular transport is the best studied form of cargo transfer between organelles of the secretory system. The different types of vesicles are classified according to the proteins making up their coat, the best studied of which are COPII, COPI, and clathrin coated vesicles. Specific proteins of the coat complex recognize cargo proteins and recruit other members to deform the membrane and bud off a vesicle. This vesicle is then actively transported along actin filaments or microtubules to the target compartment. It is brought into close proximity to the membrane by tethering factors and finally fusion is mediated by SNAREs (SNAP (Soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein) receptor) (Bonifacino and Glick, 2004). A schematic representation of the different steps of vesicular transport can be found in Figure 2.

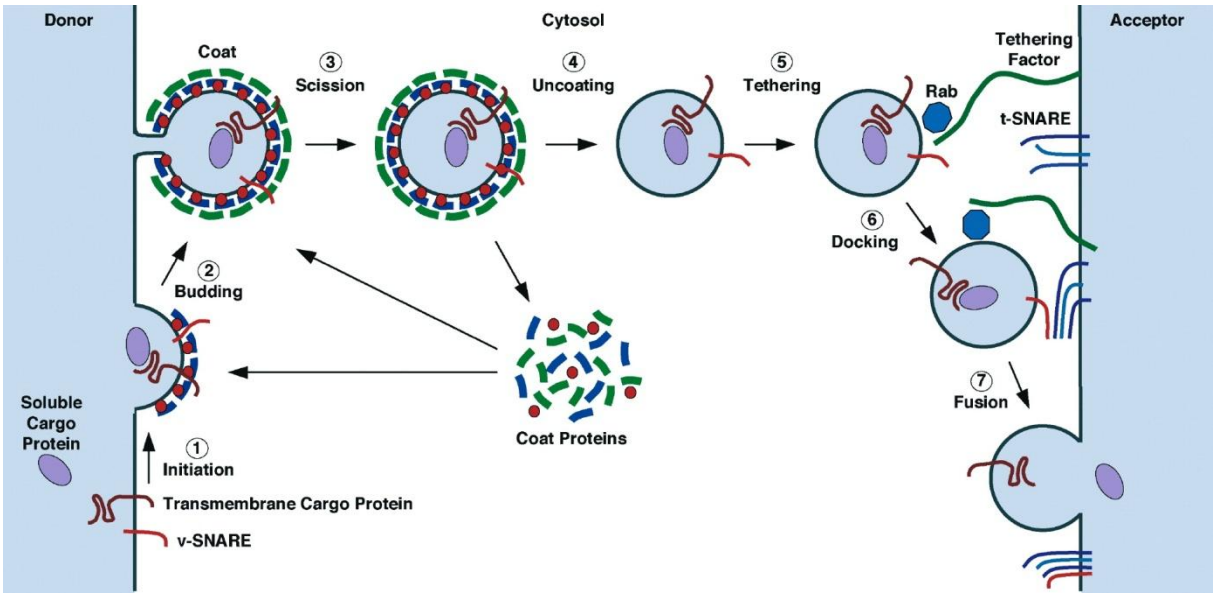

Figure 2. Steps of vesicle formation, transport, tethering, and fusion.

1. Coat assembly is initiated. A first layer of coat components (blue) is recruited to the membrane by binding to a membrane-associated GTPase (red) and/or to a specific phosphoinositide. 2. A second layer of coat components (green) is added and polymerize into a mesh-like structure. 3. The vesicle is pinched of the membrane. 4. At least partial uncoating of the vesicle takes place. 5. The vesicle is actively transported towards the acceptor compartment and is tethered close enough for fusion to occur. 6. The SNARE complex is assembled. 7. The SNARE complex promotes fusion of the vesicle with the acceptor compartment. (Reproduced from Bonifacino and Glick, 2004).

COPII Vesicles

COPII vesicles mediate transport of cargo molecules from the ER to the ERGIC/Golgi. The small GTPase Sar1 and the two protein complexes Sec23/Sec24 and Sec13/Sec31 are sufficient to generate vesicles from synthetic liposomes in the presence of nonhydrolyzable GTP analogs (Matsuoka et al., 1998). Sar1 is a small monomeric GTPase which is activated by the guanine nucleotide exchange factor (GEF) Sec12 and recruits the Sec23/Sec24 complex to the ER membrane (Weissman et al., 2001). As long as the vesicle has not yet formed, the coat is stabilized by the continuous GEF function of Sec12 which is located in ER membrane (Futai et al., 2004). Sec23 is a GTPase activating protein (GAP) for Sar1 and Sec24 interacts with transmembrane cargo proteins or cargo receptors respectively (Miller et al., 2002; Yoshihisa et al., 1993). The sorting signals recognized by Sec24 include a di-acidic ((D/E)X(D/E)) (X is any amino acid) and a di-hydrophobic motif (FF/YY/LL/FY) (Barlowe, 2003; Kappeler et al., 1997; Nishimura and Balch, 1997). There are two Sec23 and four Sec24 isoforms in mammalian cells, possibly interacting with different cargo molecules (Gurkan et al., 2006). Sec13/31 is recruited as a second layer on top of the Sar1/Sec23/Sec24 complex and drives vesicle formation (Stagg et al., 2006).

After vesicle formation the COPII coat is at least partially released due to the GAP activity of Sec23, this process probably serves to expose tethering and fusion factors buried under the coat (Lord et al., 2011; Oka and Nakano, 1994). Sec23 also directly interacts with the dynactin complex that links the COPII vesicle to microtubules along which it is then actively transported towards the ERGIC/Golgi (Watson et al., 2005). In yeast, tethering of COPII vesicles to the Golgi membrane is mediated by the TRAPPI (transport protein particle I) complex (Cai et al., 2007b). In mammalian cells TRAPPI complex and p115 are tethering factors of COPII vesicles (Allan et al., 2000; Sacher et al., 2001).

COPI Vesicles

COPI vesicles mediate the transport of proteins from the ERGIC and Golgi back to the ER, as well as intra-Golgi retrograde transport of Golgi enzymes to counteract their anterograde movement by cisternal maturation (Bonfanti et al., 1998; Letourneur et al., 1994; Martinez-Menarguez et al., 2001).

The COPI coat complex consists of eight proteins, the seven COPs (α-COP, β-COP β'- COP, γ-COP δ-COP, ε-COP, ζ-COP) and the small GTPase Arf1 (Gaynor et al., 1998). These eight components are the minimal machinery necessary to generate vesicles *in vitro* (Orci et al., 1993; Spang et al., 1998). Unlike COPII and clathrin coats, the COPI coats are preassembled in the cytoplasm and afterwards recruited *en bloc* to the membrane (Hara-Kuge et al., 1994). Several COPI components display

similarities to components of the clathrin coat, for example β-, γ-, δ- and ζ-COP resemble the AP subunits (Cosson et al., 1996; Duden et al., 1991). γ- and ζ-COP exist as two isoforms, and since only one of the components of the COPI complex is present in each complex, at least four distinguishable COPI complexes can be formed, at least three of which are physiologically relevant (Wegmann et al., 2004).

COPI vesicle formation begins with the activation of Arf1 by a GEF and subsequent recruitment of Arf1 and the COPI complex to the membrane (Zhao et al., 2002). GBF1 (Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1) was identified as the GEF involved in COPI coat formation (Kawamoto et al., 2002). Interaction with cargo or a cargo receptor containing an ER retention signal like a member of the p24 family is also required for Arf1 recruitment to Golgi membranes (Bremser et al., 1999). After the vesicle is formed, Arf1 is deactivated by the GAP ArfGAP1 which is important for vesicle uncoating (Bigay et al., 2003). The uncoated vesicle can be tethered to the ER membrane with the help of tethering factors after which SNARE mediated fusion takes place (Bethune et al., 2006).

Clathrin Coated Vesicles

Clathrin coated vesicles are used for transport steps originating at the TGN, endosomes and the plasma membrane. Similar to COPI and II, clathrin-mediated vesicle formation also includes a small GTPase which, when activated, recruits a fist layer of coat components followed by a second layer consisting of clathrin. However, since clathrin coated vesicles are involved in several distinct transport steps various adaptor proteins, linking cargo and clathrin, and additional accessory proteins are involved in forming clathrin coated vesicles at different sites and for different cargo.

Clathrin

Clathrin is a three legged structure, called a triskelion, which consists of three heavy chains and three light chains that radiate from a central hub (Ungewickell and Branton, 1981). Approximately in the middle of each of the heavy chains there is a bend introducing a swirl to the triskelion. The clathrin light chain binds to the proximal leg of the heavy chain (Kirchhausen and Toyoda, 1993). Polymerization of clathrin leads to the formation of a polyhedral cage (Kirchhausen and Harrison, 1981).

Clathrin does not directly interact with cargo proteins, but adaptor proteins are necessary as a first layer for clathrin coated vesicles to be formed. These adaptor proteins interact with the terminal

domain of the distal leg and contain a clathrin-binding motif. Clathrin-binding motives are the clathrin box with the sequence LΦXΦ(D/E) (Φ is a bulky hydrophobic amino acid, X is a polar amino acid) and the W box with the sequence PWXXW (X is any amino acid) (Dell'Angelica, 2001; Dell'Angelica et al., 1998; Miele et al., 2004).

Apart from its function in forming vesicles, clathrin has also been shown to be involved in other intracellular processes. During mitosis, clathrin is relocated to the mitotic spindle where it aids chromosomal congression (Royle et al., 2005). More recently clathrin heavy chain was shown to be required for NF-κB (Nuclear factor-κB) regulation. Constitutive activation of NF-κB could be observed when clathrin heavy chain was knocked down, but not when clathrin light chain or the adaptor protein AP-2 where knocked down (Kim et al., 2011).

Arf GTPases

The Arfs (ADP-ribosylation factors) belong to the Ras superfamily of small monomeric GTPases. Like other GTPases, they exist in an activated GTP-bound and in an inactive GDP-bound state. Arf GTPases can associate with membranes through their N-terminal myristoylation and amphiphatic helix (Amor et al., 1994; Antonny et al., 1997). In mammalian cells, there are six different Arf proteins named Arf1 through Arf6 (D'Souza-Schorey and Chavrier, 2006). Arf1 plays a role in COPI vesicle formation which has already been discussed in this thesis. However, Arf1 is also involved in the formation of clathrin coated vesicles. It has been shown that Arf1 is required for the recruitment of the adaptor proteins AP-1, AP-3, AP-4 and GGA (Golgi-localized, gamma adaptin ear-containing, Arf-binding protein), which are involved in clathrin coated vesicle formation, to the TGN (Boehm et al., 2001; Dell'Angelica et al., 2000; Ooi et al., 1998; Stamnes and Rothman, 1993). Clathrin coated vesicle formation at endosomes is also dependent on Arf1 (Pagano et al., 2004; van Dam and Stoorvogel, 2002; van Dam et al., 2002). Additionally, Arf3 has also been shown to interact with members of the GGA family (Boman et al., 2000). Next to Arf1 and 3, Arf4 and 5 are also present at the Golgi and Arf functions seem to be redundant, since knockdown of individual Arfs had no effect on Golgi function (Volpicelli-Daley et al., 2005). Arf6 can be found at the plasma membrane and has been implicated in clathrin mediated endocytosis and clathrin independent endocytosis (D'Souza-Schorey and Chavrier, 2006). Additionally, Arf6 has a function in the process of recycling from endosomes (Radhakrishna and Donaldson, 1997).

Adaptor Proteins

APs (Adaptor proteins) were the first clathrin adaptors to be identified. In mammalian cells, there are five APs named AP-1 through AP-5. AP-1, AP-3 and AP-4 localize to the TGN and endosomes with AP-3 being more endosomal and AP-4 more at the TGN, while AP-2 is found at the plasma membrane and AP-5 localizes to late endosomes (Hirst et al., 2011; Robinson, 2004). APs are heterotetrameric complexes that consist of four subunits, two large ones, a medium one and a small one. The large ones are named β1-5 and α, γ, δ, ε, or ζ, the medium one μ1-5, and the small one σ1-5 for AP1-5, respectively (Hirst et al., 2011; Owen et al., 2004; Slabicki et al., 2010).

AP-1 to AP-4 are able to directly interact with cargo proteins by binding a specific interaction motif, for example they all recognize the YXXΦ (X is any amino acid and Φ a large hydrophobic one) motif via their μ subunit (Ohno et al., 1998). AP-1 interacts with clathrin via its γ subunit and AP-2 probably via the ear domain of the β subunit (Doray and Kornfeld, 2001; Knuehl et al., 2006). AP-3 can also interact with clathrin via its β subunit but is probably not exclusively involved in clathrin dependent trafficking steps (Chapuy et al., 2008; Peden et al., 2002). Although AP-4 lacks the typical clathrin binding box, it has been suggested to interact with clathrin nonetheless (Barois and Bakke, 2005). AP-5 also lacks the clathrin binding box and probably does not associate with clathrin (Hirst et al., 2011).

AP-1 exists in two isoforms, AP-1A, which is ubiquitously expressed, and AP1-B, which is only expressed in polarized cells and has a different μ1 subunit (Ohno et al., 1999). It is involved in the formation of clathrin coated vesicles at the TGN and at endosomes (Doray et al., 2002; Pagano et al., 2004). Membrane recruitment of AP-1 involves activation of Arf1, the presence of PdtIns(4)P (phosphatidylinositol (4)-phosphate) and cargo sorting signals (Anitei and Hoflack, 2011).

AP-2 mediates clathrin coated vesicle formation in endocytosis at the plasma membrane (Traub, 2005). Membrane recruitment involves the presence of PtdIns(4,5)P_2 (phosphatidylinositol (4,5)-bisphosphate) and cargo sorting signals, recently it was shown, however, that FCH domain only (FCHo) proteins, EGFR (epidermal growth factor receptor) pathway substrate 15 (Eps15) and intersectins are also involved in AP-2 recruitment (Henne et al., 2010; McMahon and Boucrot, 2011).

AP-3 exists in two isoforms, AP-3A, which is ubiquitously expressed, and AP-3B, which is only expressed in neuroendocrine cells (Ohno, 2006). AP-3A is probably involved in trafficking cargo from the TGN to late endosomes and AP-3B in the generation of synaptic vesicles from endosomes (Nakatsu and Ohno, 2003).

AP-4 plays a role in trafficking proteins from the TGN and has been shown to be involved in basolateral sorting of the LDLR (low-density lipoprotein receptor) (Simmen et al., 2002).

AP-5 does not interact with clathrin and is independent of Arf. It was shown to be involved in trafficking of the mannose 6-phosphate receptor (Hirst et al., 2011).

Clathrin Mediated Endocytosis as an Example of Clathrin Coated Vesicle Formation

The large amount of different cargo proteins that are trafficked on different routes to and from several organelles of the late secretory pathway requires a very specific regulation of these transport processes. This regulation is in part achieved by the use of several different adaptors and accessory factors that link cargo to clathrin coated vesicles. Clathrin coated vesicles are formed at the TGN, endosomes and the plasma membrane. Clathrin mediated endocytosis is however the best studied clathrin pathway and can therefore serve as an example of the mechanism and complexity of clathrin coated vesicle formation.

The first step in clathrin coat formation at the plasma membrane is the formation of a nucleation site. The nucleation site is formed by FCH domain only (FCHo) proteins, EGFR pathway substrate 15 (Eps15) and intersectins (Henne et al., 2010; Stimpson et al., 2009). FCHos contain an F-BAR (FCH-Bin-Amphiphysin-Rvs) domain that is able to induce membrane curvature (Henne et al., 2010). Eps15 is a scaffolding protein that is able to interact with AP-2 and other endocytic protein like Epsin 1 (Salcini et al., 1999).

After formation of a nucleation site, AP-2 is recruited (Henne et al., 2010). AP-2 binds directly to PtdIns(4,5)P_2 and specific motifs in cargo proteins as well as to accessory adaptor proteins that in turn also bind to cargo molecules (McMahon and Boucrot, 2011). Accessory adaptors are adaptors that are usually cargo specific and therefore link AP-2 to cargo molecules which are not directly recognized by AP-2. For example, it could be shown that cellular AP-2 concentration can be reduced by over 90% without affecting uptake of LDL (low-density lipoprotein) (Motley et al., 2003). In contrast, this does not apply to clathrin itself (Hinrichsen et al., 2003).

Several of these adaptors that function in clathrin mediated endocytosis have been identified and termed CLASPs (clathrin-associated sorting proteins). Dab2 (Disabled 2) and ARH (autosomal recessive hypercholesterolemia) are two functionally redundant CLASPs that have been identified to function in the uptake of LDL (Garcia et al., 2001; Maurer and Cooper, 2006). Another example is stonin 2 which recruits synaptotagmin to AP-2 (Haucke and De Camilli, 1999). Finally β-arrestins have been identified to be important for the endocytosis of G protein coupled receptors (Marchese et al., 2008). Apart from these few examples many more CLASPs have been identified (McMahon and Boucrot, 2011; Schmid and McMahon, 2007; Traub, 2009).

While cargo is bound by AP-2 and additional adaptors at the nucleation site, the clathrin coat is assembled. Clathrin is recruited to the site of vesicle formation by AP-2 and can also interact with other adaptors. The polymerization of clathrin is thought to stabilize the curvature, but not to directly mediate membrane bending (McMahon and Boucrot, 2011; Saffarian et al., 2009). Membrane bending is thought to be mediated by curvature effectors that directly interact with the membrane like FCHos, Epsins and Amphiphysin (McMahon and Boucrot, 2011).

Scission of a vesicle is mediated by the mechanoenzyme dynamin that selfpolymerizes around the neck of the vesicle and undergoes a GTP hydrolysis dependent conformational change (Hinshaw and Schmid, 1995; Kosaka and Ikeda, 1983; Sweitzer and Hinshaw, 1998). Neck formation and dynamin recruitment is mediated by BAR domain-containing proteins, which have a preference for curved membranes and are able to bind dynamin. These proteins include Amphiphysin, Endophilin and Sorting nexin 9 (Ferguson et al., 2009; Sundborger et al., 2011; Wigge et al., 1997).

After vesicle scission, auxilin binds to clathrin (Massol et al., 2006). Auxilin in turn recruits HSC70 (heat shock cognate 70), an ATPase that catalyses clathrin disassembly (Schlossman et al., 1984). The activity of the phosphatase synaptojanin, which converts PtdIns(4,5)P_2 to phosphatidylinositol, is also required for uncoating (Cremona et al., 1999). A schematic representation of clathrin coated vesicle formation in endocytosis can be found in Figure 3.

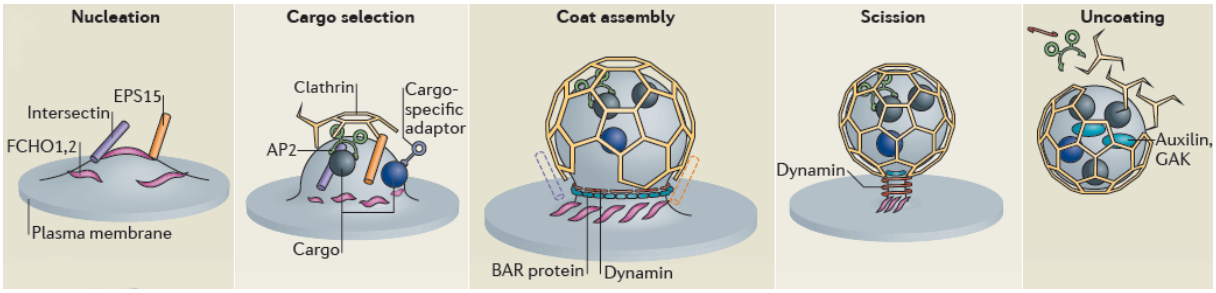

Figure 3. The proposed five steps of clathrin coated vesicle formation.

FCHo proteins bind PtdIns(4,5)P_2-rich zones of the plasma membrane, recruit Eps15 and intersectins and serve as nucleators of clathrin-coated pit formation by recruiting AP-2. AP-2 then recruits several classes of receptors directly through its μ-subunit and σ-subunit. Cargo-specific adaptors (for example stonin, HRB and Numb) bind to AP-2 appendage domains and recruit specific receptors. Clathrin triskelia are recruited by the AP-2 hub and polymerize to form the clathrin coat around the nascent pit. The GTPase dynamin is recruited at the neck of the forming vesicle by BAR domain-containing proteins and induces membrane scission to produce an endocytic vesicle. Finally, auxilin or cyclin G-associated kinase (GAK) recruits the ATPase HSC70 to disassemble the clathrin coat. (Adapted from McMahon and Boucrot, 2011).

Clathrin Independent Pathways

Apart from clathrin coated vesicles, transport in the late secretory system is also mediated by clathrin independent vesicles, tubular carriers, and organelle maturation. These clathrin independent carriers or transport mechanisms are used for transporting steps from the TGN, endosomes, and the plasma membrane. Since endocytosis was used as an example for clathrin mediated vesicle formation, endocytosis will also be used to illustrate some clathrin independent transport pathways.

Several different clathrin independent pathways are used to endocytose cargo at the plasma membrane. Caveolae are small flask shaped invaginations of the plasma membrane characterized by the presence of the integral membrane protein caveolin-1 (Parton and Simons, 2007). Caveolae are involved in cargo specific endocytosis of albumin and intergrins (Schubert et al., 2001; Shi and Sottile, 2008). After endocytosis caveolae can fuse with a compartment called the caveosome or in a Rab5 dependent manner with early endosomes (Pelkmans et al., 2004). A second clathrin independent endocytic pathway depends on the small GTPase RhoA (Lamaze et al., 2001). The pathway is used for interleukin uptake and requires RhoA and dynamin but not other key players of clathrin dependent endocytosis. Since RhoA plays a role in the regulation of the actin cytoskeleton, an involvement of the actin machinery is possible (Lamaze et al., 2001; Mayor and Pagano, 2007). A second small GTPase, Arf6, has been suggest to be important for endocytosis of class I major histocompatibility complex molecules, integrins and some other proteins (Naslavsky et al., 2004). This pathway is also dynamin independent. Finally, there is also internalization of relatively large membrane patches by macropinocytosis and phagocytosis.

Transport of Vesicles

Transport carriers are actively transported along microtubules or actin filaments from the donor to the acceptor compartment. Microtubules and actin filaments have a polarity and therefore two structurally and functionally distinct ends. Three types of motor proteins are involved in carrier transport. Kinesins generally move to the microtubule plus end, while dynein moves to the minus end. Myosins generally move towards the barbed end of the actin filament, with the exception of myosin VI that moves to the pointed end (Akhmanova and Hammer, 2010).

In order to be transported, a carrier needs to be linked to a motor protein. In general, components of the coat structure are able to interact with the motor proteins. For example, AP-1 can link the motor protein KIF13A to TGN and endosome derived carriers while AP-2 and Dab2 can link myosin VI to LDLR containing vesicles (Delevoye et al., 2009; Yu et al., 2009). Rab GTPases are important

regulatory factors that are often involved in the recruitment of motor proteins to transport carriers. For example, activated Rab8 binds to its effector optineurin which in turn binds to myosin VI, a process important in Golgi ribbon formation and exocytosis (Sahlender et al., 2005). An overview over different motor proteins involved in trafficking steps of the late secretory system is given in Figure 4.

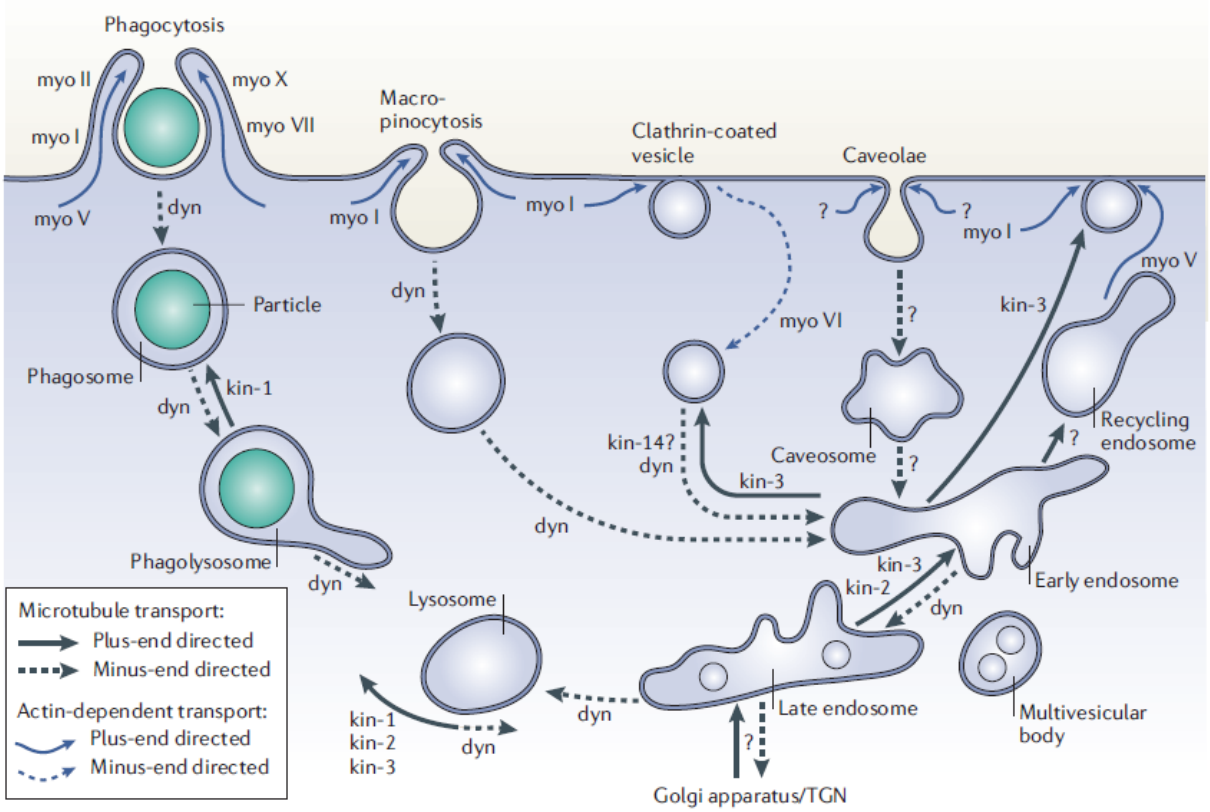

Figure 4. Overview of the motors that are involved in the late secretory system.

This figure illustrates different classes of myosin (myo), kinesin (kin) or dynein (dyn) motors that have been implicated to be involved in specific transport steps between organelles of the late secretory system. The barbed-end of actin is called plus-end in this figure, while the pointed-end is called the minus-end. (Reproduced from Soldati and Schliwa, 2006).

Tethering and Fusion of Vesicles to the Target Membrane

Following transport of a vesicle towards the target compartment, it is pulled into close vicinity of the membrane after which fusion of the vesicle with the target compartment can take place. Tethering factors bring vesicle close enough to the membrane for fusion to occur and SNARE proteins mediate actual fusion. A schematic depiction of vesicle tethering and fusion can be found in Figure 5.

Tethering factors can be subdivided into two classes, coiled-coil tethers and multisubunit tethering complexes (Sztul and Lupashin, 2006). Coiled-coil tethers usually form dimers and structurally resemble long rod-like molecules. Members of this class of tethers include p115 and EEA1 that are involved in COPII vesicle fusion and early endosomal homotypic fusion, respectively (Allan et al., 2000; Mills et al., 1998; Nelson et al., 1998). Multisubunit tethering complexes are large protein complexes consisting of several subunits. Members of these complexes include the transport protein particle (TRAPP) complex and the exocyst. TRAPP I complex consist of seven subunits, it is stably anchored in the Golgi and probably provides a tether for incoming COPII vesicles (Barrowman et al., 2000; Sacher et al., 2001). The exocyst complex consists of eight components, is located at the plasma membrane, and probably tethers incoming vesicles (TerBush et al., 1996). Rab GTPases also play an important role in tethering; for example, the yeast Rab Sec4 directly interacts with the exocyst complex (Guo et al., 1999). Apart from the Rabs, tethering factors also interact with coat components and SNAREs (Cai et al., 2007a).

The final step in the life of a vesicle is fusion with the target compartment. This fusion event is mediated by SNARE proteins. SNAREs are a family of small proteins that contain a characteristic SNARE motif and, in most cases, a C-terminal transmembrane domain (Jahn and Scheller, 2006). SNAREs are present on both membranes of the two fusion partners. Monomeric SNAREs are unstructured but as soon as two matching SNAREs are combined they associate to form a helical complex (Fasshauer, 2003). For fusion to take place, the SNAREs form a four-helical parallel bundle where every helix contains a different SNARE motif. According to the need for four different SNARE motives, SNAREs are divided into Qa-, Qb-, Qc- and R-SNAREs, whereby one of each is necessary to form a stable complex (Fasshauer et al., 1998). The free energy release by the formation of this four-helix bundle is believed to drive membrane fusion (Lin and Scheller, 1997). Following fusion, the SNARE complex needs to be disassembled. Disassembly is mediated by the ATPase N-ethylmaleimide-sensitive factor (NSF) and requires a considerable amount of energy (Sollner et al., 1993).

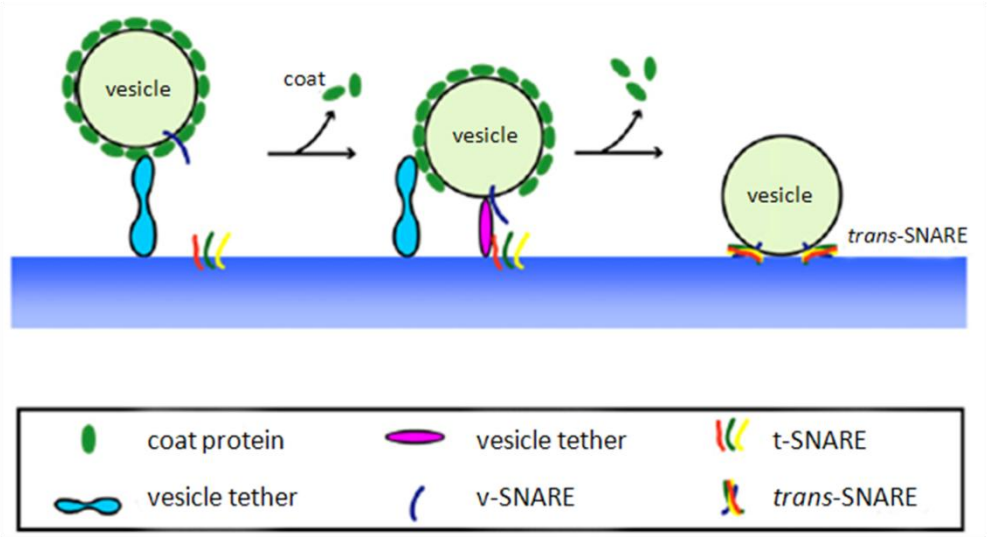

Figure 5. Tethering and fusion of vesicles.

The vesicle tether (blue) targets the vesicle to its acceptor compartment. Uncoating of the vesicle exposes the SNAREs. The exposed vesicle SNAREs then form a *trans*-SNARE pair, after which membrane fusion takes place. (Adapted from Cai et al., 2007a).

Non-vesicular Transport

Vesicles are frequently used to transfer cargo molecules from one compartment to the other. However, they are not the only means by which transport can take place, pleiomorphic, tubular-vesicular carriers are also commonly used as transport intermediates. Alternatively, an organelle may also mature, and becomes an organelle of different characteristics. In this way cargo is transported from one organelle to the next without having to be sorted into transport intermediates.

Tubular Transport

A well studied example of tubular transport is cargo sorting at the TGN. Cargo molecules leave the TGN in pleiomorphic, tubular-vesicular carriers that break into smaller pieces after they detach (Polishchuk et al., 2003). Since the TGN is a major sorting station that directs cargo molecules to various destinations, several different carriers are formed. For example in polarized cells apical and basolateral cargoes are sorted into separate carriers. Interestingly, similar cargo segregation is also observed in non-polarized cells (Kreitzer et al., 2003). A schematic representation of tubule formation at the TGN can be found in Figure 6.

Apical sorting relies on more than one mechanism. For example, N-linked and O-linked glycans are recognized and concentrated by apical sorting receptors like the lectin galectin-3 (Delacour et al.,

2007). Basolateral and endosomal cargo recognition relies on protein-protein and protein-lipid interaction and involves APs and GGAs. In polarized cells, basolateral transport is mediated by AP-1B, AP-4 and clathrin (Folsch, 2008). AP-1A, AP-3, the GGAs and clathrin mediate transport to the endosomal system (Anitei and Hoflack, 2011). The GGAs bind clathrin with different affinity and can recognize unbiquitinylated cargo molecules, probably for their transport to lysosomes and subsequent degradation (Braulke and Bonifacino, 2009).

Initial membrane curvature is mediated by Epsin N-terminal homology (ENTH) or AP180 N-terminal homology (ANTH) domain containing coat accessory proteins and clathrin (Kirchhausen, 2009; Legendre-Guillemin et al., 2004). Similar to clathrin mediated endocytosis other factors like F-BAR domain containing proteins may also play a role in membrane bending. During apical cargo sorting FAPP2 (four-phosphate adaptor protein 2) may play a role in membrane bending and tubulation (Lenoir et al., 2010).

Following curvature the membrane is elongated into tubules, a process that requires mechanical forces mediated by actin. Tubulation of basolateral carriers requires GOLPH3 (Golgi phosphoprotein 3), a protein that binds to PtdIns(4)P and the motor protein myosin XVIIIA (Dippold et al., 2009). Additionally, CDC42, its GEF FGD1, and Rac1 are involved in basolateral carrier formation (Egorov et al., 2009; Wang et al., 2005). Apical carrier tubulation is less well understood, but requires different factors like LIMK1 (LIM domain kinase 1) and cofilin, a family of actin binding proteins (Salvarezza et al., 2009).

Formation of tubular carriers transporting endosomal cargo requires Arf1 mediated recruitment of AP-1 and clathrin. A complex consisting of CYFIP (cytoplasmic FMR1-interacting protein)/Sra/Pir121 is then recruited to the membrane. Rac1 is activated by its GEF β-PIX (PAK-interacting exchange factor) that binds to CYFIP and promotes N-WASP (neural Wiskott-Aldrich syndrome protein) and Arp2/3-dependent actin polymerization (Anitei et al., 2010). BAR domain containing proteins may be involved in stabilizing tubular membranes (Wu et al., 2010). Actin-based myosin motor proteins play a role in tubule elongation and fission. For example the Rab6 effector myosin II is believed to promote fission of tubular carriers (Miserey-Lenkei et al., 2010). Additionally, several microtubule based kinesin motors have also been implicated in tubule elongation and carrier fission at the TGN (Anitei and Hoflack, 2011).

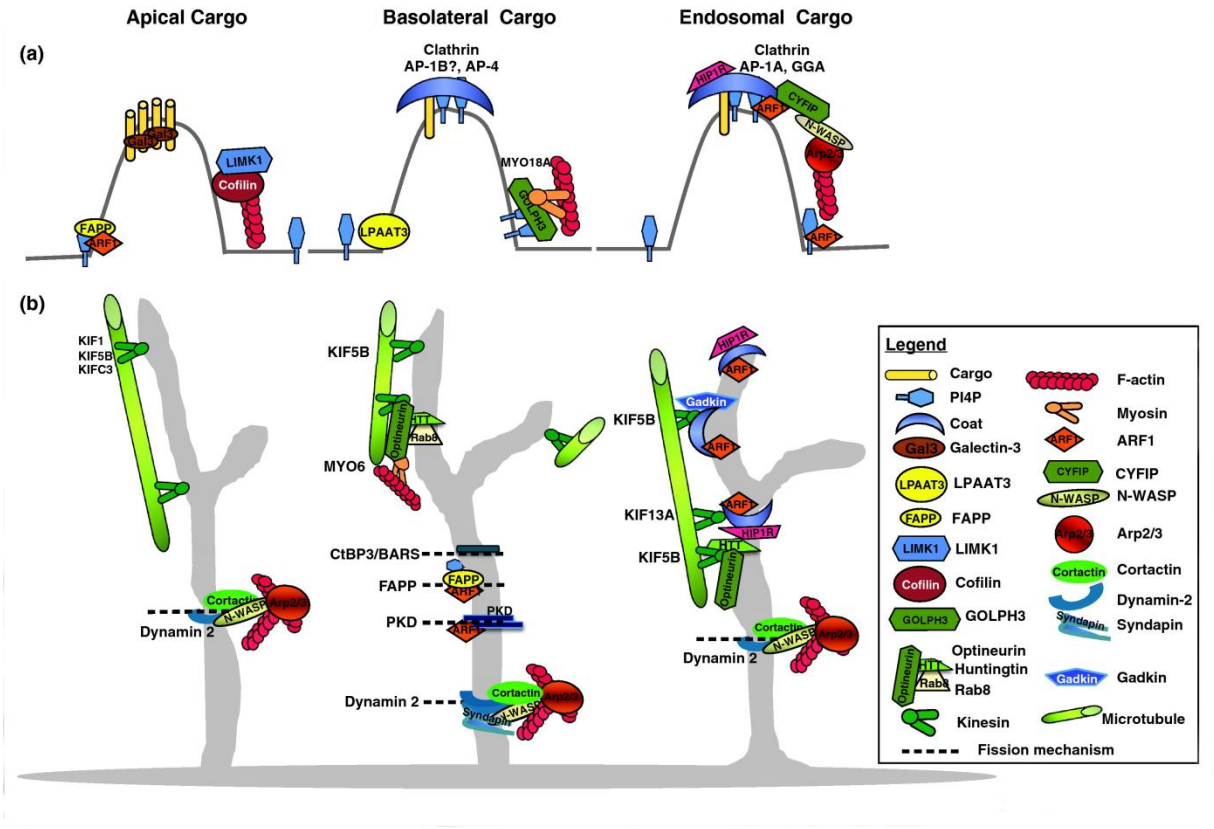

Figure 6. Transport carrier formation at the TGN.

(A) During the early stages of carrier formation, non-coated apical, coated basolateral, or coated endosomal carriers bud from the TGN membrane.
(B) Tubule formation depends on microtubules, actin filaments and motor proteins among other factors. (Reproduced from Anitei and Hoflack, 2011).

Organelle Maturation

Some organelles gradually change their identity, a process called maturation, for example by exchanging organelle associated proteins and altering the luminal pH. Cargo molecules do not need to leave the lumen of the organelle in the process. Examples of organelle maturation include intra-Golgi anterograde transport and the maturation of early sorting endosomes into late and recycling endosomes.

A prominent example of organelle maturation is the maturation of the globular part of the early sorting endosomes into late endosomes/multivesicular bodies and subsequently into lysosomes. Transition from one organelle to another coincides with the exchange of organelle associated proteins that are specific for a certain organelle. For example, the Rab5 containing early sorting endosomes mature into Rab7 containing late endosomes (Rink et al., 2005). Rab7 is recruited to endosomes by the HOPS (homotypic fusion and vacuole protein sorting) complex. One of the subunits of the HOPS complex, Vps39, is a GEF for Rab7 (Wurmser et al., 2000). A study in *C. elegans*

coelomocytes has shown a mechanism for early sorting to late endosome maturation. SAND-1/Mon1 breaks the positive feedback loop of Rab5 recruitment to early sorting endosomes by displacing the Rab5 GEF Rabex-5. Furthermore, SAND-1/Mon1 can interact with the HOPS complex and thereby recruits Rab7 to the membranes (Poteryaev et al., 2010).

The Recycling System

Cells constantly internalize extracellular material like plasma membrane proteins or ligands via clathrin dependent and independent endocytosis. To counteract membrane loss due to endocytosis the endosmal recycling system returns much of the endocytosed membrane portions and proteins back to the cell surface. Endocytosed cargo is typically delivered to early sorting endosomes. From early sorting endosomes it can be routed to late endosomes and consequently lysosomes, to the TGN, or back to the plasma membrane, either directly or via recycling endosomes (Grant and Donaldson, 2009; Jovic et al., 2010). Moreover, recycling does not only occur via two distinct populations of endosomes, but transport receptors like TfR, LDLR, and the asialoglycoprotein receptor are recycled in a constitutive fashion, while signaling receptors like the β2 adrenergic receptor and EGFR, or other proteins like MHC (major histocompatibility complex) class I and II molecules, and β-integrins are recycled in a regulated fashion.

A number of proteins have been identified to be involved in the recycling system, but the mechanism of recycling from endosomes is only poorly understood. Many of the identified proteins are members of the small monomeric GTPase families Rab and Arf, the EHD (receptor mediated endocytosis 1 family of carboxy-terminal epidermal growth factor receptor substrate 15 homology domain-containing) protein family and motor proteins (Grant and Caplan, 2008; Grant and Donaldson, 2009). The function of Rab proteins, also with respect to their role in recycling, will mainly be discussed in the following chapter. An overview over the pathways of the recycling system and some of the involved factors can be found in Figure 7.

Direct recycling from early sorting endosomes to the plasma membrane is commonly termed “fast recycling”. Rab4 has been implicated to be important for fast recycling of the TfR, however the published literature is not clear on the precise role of Rab4 (Deneka et al., 2003; McCaffrey et al., 2001; Mohrmann et al., 2002b; van der Sluijs et al., 1992; Yamamoto et al., 2010). Clathrin coated vesicles have been shown to be formed at early endosomes in a dynamin dependent fashion, and in an *in vitro* assay, the formation of recycling vesicles containing the asialoglycoprotein receptor H1 has benn shown to require Rab4, AP-1 and clathrin (Pagano et al., 2004; van Dam and Stoorvogel, 2002). Furthermore, the Rab4 effector Rabaptin-5 also contains a binding site for AP-1, thereby

linking AP-1 to early sorting endosomes (Deneka et al., 2003). Rab35, another member of the Rab family, has also been implicated to be required for rapid recycling of the TfR (Kouranti et al., 2006).

Instead of being directly transported from early sorting endosomes to the plasma membrane, cargo proteins can also take a longer route, commonly termed “slow recycling”, via perinuclear localized recycling endosomes. It is commonly thought that the early sorting endosome extends tubules that pinch off and mature into recycling endosomes, while the globular part of the early sorting endosomes matures into late endosomes (Maxfield and McGraw, 2004).

Transport of cargo proteins from early sorting endosomes to recycling endosomes is dependent on several proteins. EHD4 is involved in cargo transport from early sorting endosomes towards both the recycling endosomes and late endosomes (Sharma et al., 2008). The closely related EHD3 interacts with the two Rab effectors Rabenosyn-5 and Rab11-FIP2, indicating a role for EHD3 as a linker between early sorting and recycling endosomes (Naslavsky et al., 2006). The Rab11 effector Rab11-FIP5 is required for transport of the TfR to recycling endosomes. Without Rab11-FIP5, TfR is only recycled from early sorting endosomes (Schonteich et al., 2008). Another Rab11 effector, Rab11-FIP3, together with its binding partner ASAP1 (Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1) is important for correct positioning of recycling endosomes in the perinuclear region (Inoue et al., 2008). Additionally, the two Rabs Rab22a and, in polarized cells, Rab10 have been implicated to be important for cargo transport between early sorting and recycling endosomes (Babbey et al., 2006; Magadan et al., 2006). Sorting nexin 4 is important to transport the TfR from early sorting to recycling endosomes. Without sorting nexin 4 the TfR is delivered to late endosomes and consequently degraded in lysosomes. Sorting nexin 4 also indirectly interacts with the motor protein dynein, suggesting a role in the correct localization of recycling endosomes (Traer et al., 2007).

Transport from recycling endosomes to the plasma membrane is probably mediated by more than one pathway. For example, cargo internalized via clathrin dependent or independent pathways is also segregated into differing recycling pathways (Grant and Donaldson, 2009). An important protein in the regulation of recycling from perinuclear recycling endosomes is Arf6 (Radhakrishna and Donaldson, 1997). For example, recycling of syndecan-1 and FGFR (fibroblast growth factor receptor) requires Arf6, PtdIns(4,5)P_2, and syntenin, a protein that can bind the C-terminus of syndecans (Zimmermann et al., 2005). PtdIns(4,5)P_2 is produced by PI4P5 kinase, an enzyme that is activated at recycling endosomes by Arf6 (Brown et al., 2001). Another major player in the transport of cargo from recycling endosomes to the plasma membrane is Rab11, since Rab11 was suggested to regulate slow recycling of the TfR and recycling of MHC class I and β-integrin (Powelka et al., 2004; Ullrich et al., 1996; Weigert et al., 2004). Recycling of MHC class I molecules is also dependent on Rab22a and

EHD1 (Caplan et al., 2002; Weigert et al., 2004). Clathrin mediated recycling uses ACAP1 (ArfGAP with coiled-coil, ANK (Ankyrin) repeat and pleckstrin homology domain-containing protein 1) as an adaptor protein (Li et al., 2007). ACAP1 is also a GAP for Arf6, suggesting a role for Arf6 in clathrin mediated recycling (Jackson et al., 2000). In polarized cells the adaptor protein AP-1B and Rab8 are required for clathrin mediated recycling to the basolateral surface (Ang et al., 2003; Gan et al., 2002). EHD1 and Amphiphysin may act in conjunction to promote vesicle fission from recycling endosomes (Pant et al., 2009).

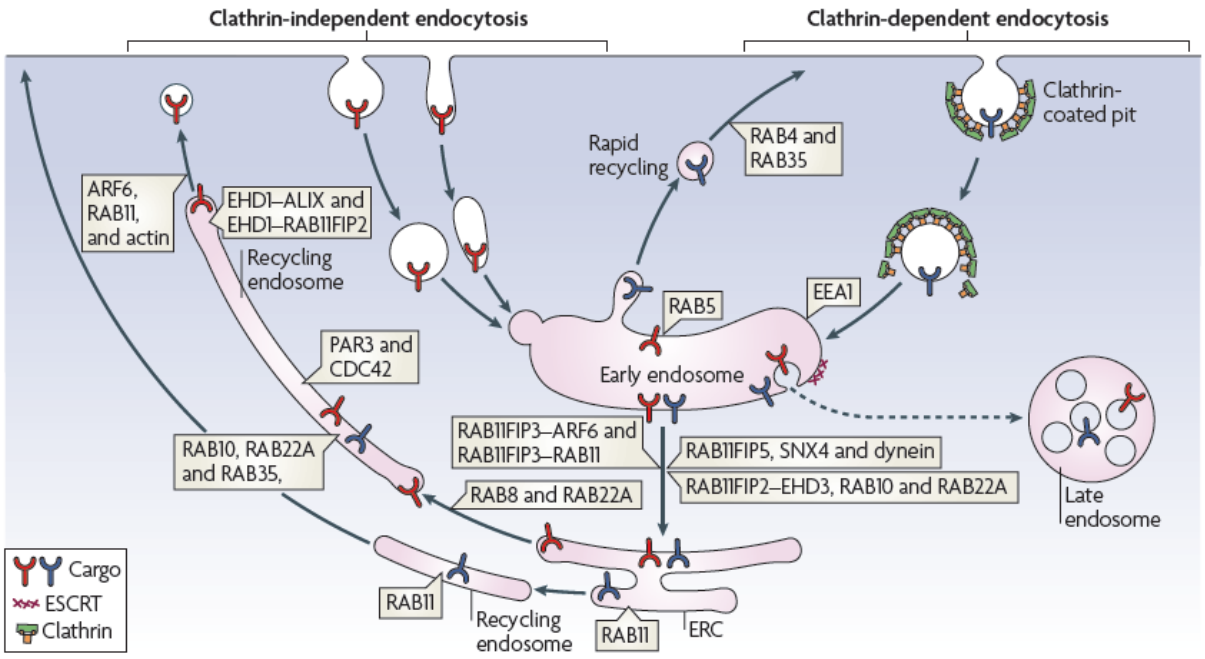

Figure 7. Pathways of endocytosis and endocytic recycling.

This figure shows the itinerary of cargo proteins entering cells by clathrin-dependent (blue cargo) and clathrin-independent (red cargo) endocytosis. Clathrin-dependent cargoes can recycle back to the cell surface through a rapid recycling pathway that may involve Rab4 and Rab35. Both types of cargo can move from early endosomes to recycling endosomes by a process that requires sorting nexin 4, dynein, Rab10, Rab22A, Rab11-FIP2 in complex with EHD3, Rab11-FIP3 in complex with Arf6, or Rab11 and Rab11-FIP5. From recycling endosomes, recycling of some types of cargo may require Rab11, and recycling of clathrin-independent cargoes involves the generation of distinctive Rab8- and Rab22A-dependent tubules, in addition to many other factors. Some clathrin-dependent cargoes might also recycle through these different pathways (Reproduced from Grant and Donaldson, 2009).

Rab GTPases

Rab proteins are a group of small monomeric GTPases with over 70 members in mammalian cells (Colicelli, 2004). An overview of the localization and presumed function of some Rabs is presented in Figure 8. Rab proteins are molecular switches, cycling between an active GTP bound and an inactive GDP bound state. GDP to GTP exchange is catalyzed by GEFs (guanine nucleotide exchange factors), which facilitate GDP release. GTP hydrolysis is driven by the intrinsic GTPase activity of the Rab protein and by GAPs (GTPase activating proteins) (Stenmark, 2009). Normally, two geranylgerany groups are attached to two cysteines at the C-terminus, through which Rabs can be reversibly associated with membranes. Upstream of the geranylgerany groups there is a hypervariable region that differs more between Rabs than other regions, and plays a role in correct membrane targeting. Interchange of hypervariable domains leads to the mislocalization of Rabs (Chavrier et al., 1991). Additionally, conserved stretches of amino acids were identified named F1-F5 and SF1-SF4 that allowed the Rabs to be classified into families and subfamilies respectively (Pereira-Leal and Seabra, 2000). These F and SF regions have also been shown to be important for membrane targeting (Ali and Seabra, 2005).

Following translation, a Rab protein is bound by REP (Rab escort protein), which presents the Rab to the RabGGT (Rab geranylgeranyl transferase). The RabGGT covalently attaches one or two geranylgeranyl lipid groups to the C-terminus of the Rab protein (Alexandrov et al., 1994; Desnoyers et al., 1996). Cytosolic Rab proteins are bound by GDI (Rab-GDP dissociation inhibitor), a protein that prevents GDP release from the Rab, aids in correct membrane localization of the Rab, and extracts GDP-bound Rabs from the membrane (Matsui et al., 1990; Ullrich et al., 1994; Ullrich et al., 1993). Another protein, GDF (GDI dissociation factor), promotes GDI dissociation from the Rab protein and thereby helps the Rab to associate with the correct membrane (Sivars et al., 2003).

The process of a transport reaction can be divided into five steps: The formation of a transport intermediate (e.g vesicle), its movement to the target compartment, partial uncoating of the transport intermediate, tethering to the membrane of the target compartment, and finally fusion with the target compartment. Rab GTPases have been shown to be involved in the regulation of each of these five steps in several of the different intracellular transport routes (Grosshans et al., 2006; Hutagalung and Novick, 2011; Schwartz et al., 2007; Stenmark, 2009). Some examples of the function of Rabs in these five steps are described below.

Formation of carriers. Several Rab proteins have been shown to play a role in the process of cargo selection or vesicle formation. A well characterized example is that of Rab9. Rab9 localizes to late

endosomes where it recruits its effector TIP47, which interacts with the mannose-6-phosphate receptor and is required for the transport of mannose-6-phosphate receptor from late endosomes to the TGN (Diaz and Pfeffer, 1998; Lombardi et al., 1993). Another example is that of Rab5, which in complex with GDI is required for sequestration of transferrin receptor into clathrin coated pits at the plasma membrane (McLauchlan et al., 1998). Furthermore, Rab4 has been shown to play a significant role as a regulator of AP-1 dependent clathrin coated vesicle formation at endosomes (Pagano et al., 2004).

Motility of carriers. Rab GTPases also recruit effectors that are relevant for the movement of vesicles along microtubules and actin filaments. For example, Rab27a recruits its effector melanophilic/Slac2a to melanosomes, the melanin containing organelles of melanocytes, which in turn recruits myosin Va (Strom et al., 2002). Motor proteins can also directly interact with Rab GTPases, for example, the kinesin motor Rabkinesin-6 has been shown to be an effector of Rab6 (Echard et al., 1998).

Carrier uncoating. In order for a vesicle to be able to fuse with the membrane of the target compartment the vesicular coat must be shed. Rab GTPases also participate in this vesicular uncoating process. Rab5 plays an important role in the uncoating of endocytic clathrin coated vesicles. AP-2, a major component of the endocytic clathrin coated vesicle coat, needs to be phosphorylated at its µ2 subunit to interact with cargo molecules and requires PtdIns(4,5)P_2 to recognize sorting signals. The Rab5 GEF GAPVD1 displaces the kinase that phosphorylates µ2 and Rab5 alters PtdIns(4,5)P_2 levels in the membrane by recruiting its effectors phosphatidylinositol 3-kinase and phosphatidylinositol phosphatases (Semerdjieva et al., 2008; Shin et al., 2005).

Tethering. Tethering of vesicles to the target membrane is an important prerequisite for fusion. Tethering is mediated by tethering factors, many of which are Rab effectors. For example the coiled-coil tethering factor EEA1 is an effector of Rab5 and important for homotypic fusion of early endosomes (Mills et al., 1998). In yeast, the large tethering complex exocyst mediates tethering of secretory vesicles to the plasma membrane. One of the exocyst subunits has been shown to directly interact with the yeast Rab Sec4p (Guo et al., 1999).

Fusion. Membrane fusion is the last step in the life of a transport vesicle and is mediated by the action of SNARE proteins. Rab5 is indirectly crucial for fusion, since its effectors EEA1 and rabenosyn-5 interact with the SNARE protein syntaxin-6 and the SM protein VPS45, respectively (Nielsen et al., 2000; Simonsen et al., 1999).

The function of Rab proteins goes beyond an actual role in transport, they are also involved in organelle morphology and membrane identity. Membrane identity is specified by recruiting effectors

and controlling phosphoinositide levels through phosphoinositide kinases and phosphatases. For example, the Inositol polyphosphate 5-phosphatase OCRL-1 is an effector of several Rabs and Rab5 recruits PI3 kinase to early sorting endosomes (Christoforidis et al., 1999; Fukuda et al., 2008). Some organelles mature into different compartments by interchanging its protein and lipid composition. Organelles that are defined by a Rab protein need to exchange one Rab for another. In the most prevalent model, an upstream Rab will recruit the GEF for a downstream Rab. This downstream Rab will then in turn recruit the GAP for the upstream Rab, thereby effectively replacing it. For example, early sorting endosomes mature into late endosomes and exchange Rab5 for Rab7 along the way (Rink et al., 2005). In *C. elegans* coelomocytes it was shown that SAND-1/Mon1 breaks the positive feedback loop of Rab5 recruitment to early sorting endosomes by displacing the Rab5 GEF Rabex-5. SAND-1/Mon1 then recruits Rab7 to the membranes, probably by interacting with the HOPS complex, a GEF for Rab7 (Poteryaev et al., 2010).

To emphasize the role of Rabs in endocytic recycling, the following chapters will deal with Rab4, Rab11, Rab15, Rab35, and Rab14, all of which have at least been implicated to be involved in the recycling route.

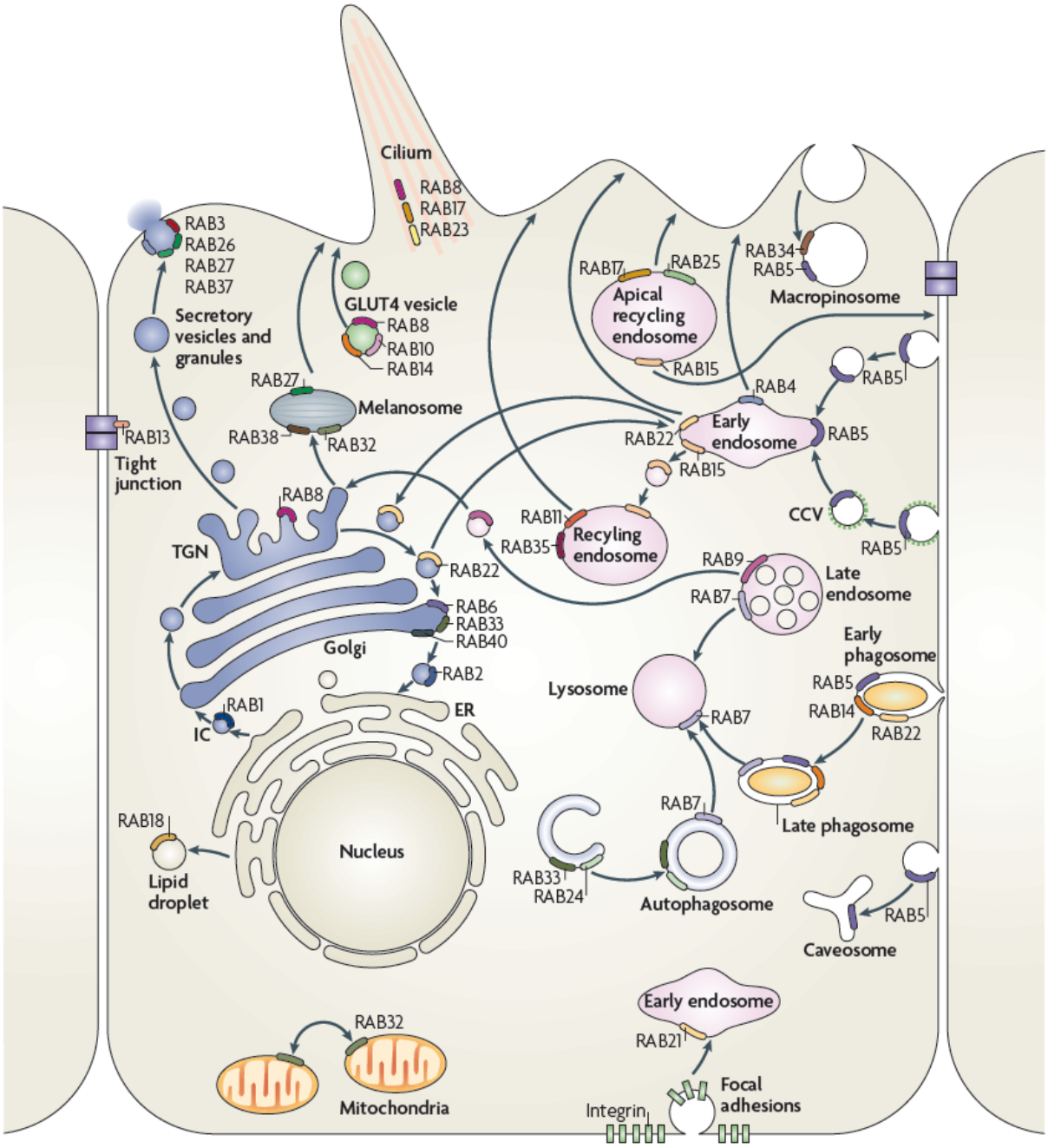

Figure 8. Localization and presumed function of several Rab proteins.

Over 70 Rab proteins have been identified. They localize to virtually every organelle of the secretory system and additionally to other specific sites or organelles within a cell. Rabs are involved in the formation of transport intermediates, the active transport of such intermediates along microtubules and actin filaments, as well as in tethering and fusion of transport intermediates to the target compartment (Reproduced from Stenmark, 2009).

Rab4

Rab4 has been shown to localize to early sorting endosomes in CHO and A431 cells, where it co-localizes with transferrin-(Tf)-positive structures and partially overlaps with EEA1 (Daro et al., 1996; Sonnichsen et al., 2000; Van Der Sluijs et al., 1991).

The role of Rab4 in recycling from endosomes to the plasma membrane is not entirely clear. In an early study Rab4 was shown to affect the cellular distribution of the TfR in CHO cells (van der Sluijs et al., 1992). Overexpression of wild type, dominant negative, or constitutively active Rab4 shifted the steady state distribution of the TfR towards the plasma membrane side. However, the return rate of the TfR to the plasma membrane was unaffected by any of these mutants. Additionally, in polarized MDCK cells Rab4 was shown to have no effect on recycling of the TfR, but overexpression of wild-type and constitutively active Rab4 caused redistribution of TfR from the basolateral to the apical surface and enhanced Tf transcytosis (Mohrmann et al., 2002b). Additionally, it could be shown that by anchoring Rab4 to early endosomes, disrupting its membrane-cytosol cycle, Tf transcytosis to the apical membrane was slightly reduced, while recycling to the basolateral membrane was unaffected (Mohrmann et al., 2002a). In contrast to these findings, the fluid phase marker horseradish peroxidase was removed faster from CHO cells expressing dominant negative Rab4 (van der Sluijs et al., 1992). Furthermore, dominant negative Rab4 was mentioned to strongly impair recycling of Tf, low-density lipoprotein (LDL) and epidermal growth factor (EGF) in HeLa cells (data not shown in McCaffrey et al., 2001). Interestingly, siRNA mediated knockdown of Rab4 in HeLa cells appeared to enhanced the recycling rate of the TfR in one study while it decreased the recycling rate in another (Deneka et al., 2003; Yamamoto et al., 2010).

There is *in vitro* evidence for an involvement of Rab4 in the formation of transport carriers at early sorting endosomes. It could be shown, that large early endosomes which were created by Rab5 mediated homotypic fusion, undergo fission that is stimulated by wild-type and constitutively active, but not by dominant negative Rab4 (Chavrier et al., 1997). Furthermore, Rab4 regulates the formation of synaptic-like microvesicles at early endosomes in PC12 cells. Overexpression of dominant negative Rab4 inhibited formation of Tf containing, and synaptic-like microvesicles at early endosomes (de Wit et al., 1999; de Wit et al., 2001). It could also be shown that Rab4 is necessary to generate recycling vesicles containing asialoglycoprotein receptor subunit H1 at early endosomes (Pagano et al., 2004).

To exert its function as a molecular switch, Rab4 interacts with several effector proteins. Some of these effectors have an influence on recycling, reinforcing the notion that Rab4 is somehow involved. For example, Rab4 directly interacts with Rabaptin-5α, a splice variant of Rabaptin-5 also known as

Rabaptin-4, and was shown to recruit it to the endosomal membrane (Nagelkerken et al., 2000; Vitale et al., 1998). Rab4 co-localizes with Rabaptin-5α-positive early sorting endosomes, and overexpression of Rabaptin-5α slowed Tf recycling in HeLa cells (Deneka et al., 2003). Since a study showed that Rabaptin-5α co-localizes with AP-1 and binds its γ-subunit, while another study showed that Rab4, AP-1 and clathrin are important for carrier formation at endosomes, it is highly likely that Rab4 regulates clathrin coated vesicle formation at endosomes (Deneka et al., 2003; Pagano et al., 2004).

Rabenosyn-5 is another direct interactor of Rab4 and Rab5 implicated to be involved in recycling. Rabenosyn-5 contains a FYVE finger domain that mediates binding to PtdIns(3)P, and localizes to early sorting endosomes. Overexpression of Rabenosyn-5 enhanced transport of Tf from early sorting endosomes to the plasma membrane, while it inhibited transport to recycling endosomes in HeLa cells (de Renzis et al., 2002; Nielsen et al., 2000).

Rabip-4 is an effector of Rab4 and Rab14. It contains a FYVE finger domain that mediated binding to PtdIns(3)P, and localizes to early sorting endosomes. Overexpression of Rabip-4 led to the enlargement of endosomes, an effect that was enhanced by simultaneous overexpression of active Rab4 and counteracted by dominant negative Rab4 (Cormont et al., 2001; Yamamoto et al., 2010).

The Rabip-4 variant Rabip-4' also localizes to early sorting endosomes, where it co-localizes with Tf and EEA1, and binds Rab4. Overexpression of a dominant negative version of Rabip4' led to reduced uptake and strong impairment of Tf recycling in HeLa cells. The effect on uptake is probably linked to the fact that Rabip4' also binds Rab5. Rabip4' may therefore be connecting endocytosis with recycling (Fouraux et al., 2004).

D-AKAP2 (Dual-specific A-kinase-anchoring protein 2) can interact with protein kinase A and PDZ domain containing proteins, and contains two tandem RGS (regulator of G-protein signaling) domains, which usually interact only with heterotrimeric G proteins. These RGS domains were shown to interact with Rab11 and Rab4. A fraction of the normally cytosolic D-AKAP2 localizes to endosomes, where it was recruited by Rab4 and Rab11. Knockdown of D-AKAP2 by siRNA was shown to increase the recycling rate of Tf in HeLa cells (Eggers et al., 2009).

Recently the neuron specific Rab4 effector GRASP-1 (GRIP (glutamate receptor interacting protein)-associated protein-1) was identified. GRASP-1 was shown to be necessary for AMPA receptor recycling and coordination of the connection of Rab4 positive and Rab11 positive endosomes via interaction with the SNARE syntaxin 13 (Hoogenraad et al., 2010).

Since Rab4 interacts with dynein light intermediate chain 1, a microtubule minus end motor protein, and because its effectors D-AKAP2 and GRASP-1 also interact with Rab11, Rab4 mighty play a role in the transport step from early sorting to recycling endosomes (Bielli et al., 2001; Eggers et al., 2009; Hoogenraad et al., 2010).

Next to the formation of transport vesicles, Rab4 was also shown to play a potential role in the fusion of GLUT4 storage vesicles with the plasma membrane, since Rab4 recruited syntaxin-4 to GLUT4 containing vesicles. Syntaxin-4 is a SNARE protein necessary for GLUT4 vesicle fusion with the plasma membrane (Li et al., 2001).

Rab11

Rab11 localizes to perinuclear recycling endosomes in non-polarized and in polarized cells (Casanova et al., 1999; Green et al., 1997; Ullrich et al., 1996). Furthermore, Rab11 has been reported to localize to the TGN (Chen et al., 1998; Urbe et al., 1993).

In non-polarized cells, Rab11 was implicated in the transport of cargo from recycling endosomes to the plasma membrane. Recycling of the TfR was shown to be reduced in CHO and BHK cells overexpressing dominant negative Rab11. Wild type and constitutively active mutants of Rab11 also reduced recycling albeit to a lesser extent (Ren et al., 1998; Ullrich et al., 1996). Apart from the TfR, Rab11 also has been shown to play a role in the recycling of several signaling receptors, including β1 integrin, β2-adrenergic receptors and transforming growth factor-β (Mitchell et al., 2004; Moore et al., 2004; Powelka et al., 2004).

Interestingly, in polarized cells Rab11 was found to be involved in recycling of apical cargo, while recycling of the TfR to the basolateral membrane was independent of Rab11. In cells overexpressing dominant negative Rab11 recycling of IgA to the apical side was impaired (Casanova et al., 1999; Wang et al., 2000).

Rab11 interacts with several effector proteins termed Rab11-FIPs (Rab11-family interacting proteins, also known as FIPs). The FIPs are divided into two classes, class 1 FIPs include Rip11 (also known as FIP5), FIP2 and RCP (Rab coupling protein, also known as FIP1C) while class 2 FIPs include FIP3 and FIP4 (Horgan and McCaffrey, 2009). Some of these FIPs have been shown to be involved in the process of recycling. For example, overexpression of a truncated form of Rip11 led to moderately impaired apical IgA recycling and had no effect on basolateral Tf recycling in MDCK cells, similar to the effect of dominant negative Rab11 (Prekeris et al., 2000). Additionally, overexpression of a truncated form of RCP caused significant reduction of Tf recycling in HeLa cells (Lindsay et al., 2002).

Surprisingly, RNAi mediated knockdown of RCP had no effect on TfR and EGFR recycling, instead TfR was misrouted to the lysosomes (Peden et al., 2004). Furthermore, FIP2 was shown to be involved in recycling of aquaporin-2 and CXC chemokine receptor 2. Recycling of both proteins was slowed in cells overexpressing a truncated form of FIP2 (Fan et al., 2004; Nedvetsky et al., 2007). Another Rab11 effector Rab11BP/Rabphillin-11 has also been reported to play a role in TfR recycling, since overexpression of a truncated form of Rab11BP/Rabphillin-11 that still can bind to Rab11 impaired recycling to the same extent as dominant negative Rab11 in CHO cells (Zeng et al., 1999).

Several findings have implicated Rab11 in the transport pathway from sorting endosomes to recycling endosomes. First of all, overexpression of dominant negative Rab11 diminished accumulation of Tf in the perinuclear region, while wild-type and constitutively active promoted accumulation of Tf there (Ren et al., 1998). Additionally, the Rab11 effector FIP2 interacts with EHD1. Loss of EHD1 expression prevented delivery of the TfR from early sorting endosomes to recycling endosomes (Naslavsky et al., 2006). Furthermore, Rab11 is linked to the motor protein dynein via the Rab11 effector FIP3. This interaction is necessary for the transport of cargo from sorting endosomes to recycling endosomes and the correct perinuclear positioning of recycling endosomes (Horgan et al., 2010).

The function of Rab11 at the TGN is somewhat vague. So far it has been shown that overexpression of dominant negative Rab11 caused vesicular stomatitis virus glycoprotein to accumulate at the Golgi, while wild-type and constitutively active Rab11 had no effect (Chen et al., 1998). Furthermore, phosphatidylinositol 4-kinase β recruited Rab11 to the Golgi and a functional interaction between the two was necessary for the vesicular stomatitis virus glycoprotein to exit the Golgi (de Graaf et al., 2004). Another study found that overexpression of wild-type, dominant negative and constitutively active Rab11 inhibited shiga toxin B-subunit and TGN38 transport from endosomes to the TGN (Wilcke et al., 2000).

Rab11 may also have a function in the transport of carrier intermediates from endosomes to the plasma membrane. The Rab11 effector FIP2 directly interacts with myosin Vb and interference with this interaction led to delayed Tf recycling in HeLa cells and delayed IgA trafficking in MDCK cells (Hales et al., 2002).

Rab15

Rab15 has been shown to localize to peripheral punctae and the perinuclear region in Tf positive structures. Subcellular fractionation and organelle immunoisolation confirmed colocalisation with Tf, Rab4, Rab5 and Rab11 positive structures indicating that Rab15 resides on sorting endosomes and recycling endosomes (Zuk and Elferink, 1999).

Rab15 was shown to play a role in the endocytosis of Tf by regulating homotypic early endosome fusion. Constitutively active Rab15 mutants inhibited fusion of early endosomes and slowed endocytosis, while inactive mutants promoted fusion and increased the relative amount of endocytosed Tf (Zuk and Elferink, 2000).

Rab15 has also been implicated in Tf recycling from endosomes. In CHO cells, recycling from recycling endosomes was inhibited by the constitutively GDP-bound mutant and a mutant unable to bind a guanine nucleotide, while recycling from early sorting endosomes was only inhibited by the constitutively GDP-bound mutant of Rab15. Wild type and constitutively GTP-bound Rab15 did not influence recycling (Zuk and Elferink, 2000). The impact on recycling and homotypic early endosome fusion by Rab15 is modulated by the effector protein Mammalian Suppressor of Sec4 (Mss4) (Strick et al., 2002).

REP15 (Rab effector protein 15) was identified as a direct interactor of Rab15. REP15 and Rab15 co-localize on recycling endosomes but REP15 is absent from sorting endosomes. REP15 was implicated to be involved in the regulation of Tf recycling, since overexpression and knockdown of REP15 inhibited recycling from recycling endosomes but not from sorting endosomes (Strick and Elferink, 2005).

Rab35

Rab35 can be found at the plasma membrane where it is concentrated at clathrin coated pits, endocytic clathrin coated vesicles, and sorting endosomes (Kouranti et al., 2006). Rab35 was shown to be recruited to clathrin coated pits via its GEF connecdenn-1, a DENN (differentially expressed in normal and neoplastic cells) domain containing protein that interacts with clathrin and AP-2 (Allaire et al., 2010; Marat and McPherson, 2010).

Several studies have reported Rab35 to be important for receptor recycling from early endosomes to the plasma membrane. Overexpression of dominant negative Rab35 impaired Tf recycling while the constitutive active mutant increased the recycling rate in HeLa cells. Additionally, the amount of TfR

at the plasma membrane was reduced in cells expressing the dominant negative mutant (Kouranti et al., 2006). Similarly, Rab35 was shown to be important for yolk receptor recycling in *C. elegans* oocytes, T-cell receptor and Tf recycling in Jurkat cells and major histocompatibility complex class I recycling (Allaire et al., 2010; Patino-Lopez et al., 2008; Sato et al., 2008).

However, one study failed to reproduce the importance of Rab35 for Tf recycling in Cos-7 and HeLa cells by the use of Rab35 knockdown and dominant negative mutants, suggesting that Rab35 functions in clathrin independent recycling (Allaire et al., 2010). Indeed, MICAL-L1, a protein that functions together with EHD-1 and Arf6 in clathrin independent recycling, was identified as a Rab35 interaction partner (Sharma et al., 2010; Sharma et al., 2009). Interestingly, a recent study found Arf6 to negatively regulate Rab35 activity via its effector EPI64B, which is also a GAP for Rab35 (Chesneau et al., 2012).

Rab14

Rab14 is a ubiquitously expressed protein that mainly localizes to the TGN and endosomes and to recycling endosomes in polarized cells (Junutula et al., 2004; Kelly et al., 2010; Kitt et al., 2008; Proikas-Cezanne et al., 2006).

Rab14 is believed to be involved in transport steps from the TGN to endosomes, because the localization of dominant negative Rab14 is shifted towards the TGN, while in case of constitutively active Rab14 it is shifted towards the endosomes (Junutula et al., 2004). Indeed Rab14 has been shown to be involved in the transport of the fibroblast growth factor receptor from the TGN to endosomes (Ueno et al., 2011). Furthermore, in polarized cells, Rab14 plays a role in apical sorting of cargo from the TGN, since overexpression of dominant negative Rab14 mislocalized apical cargo to the basolateral surface (Kitt et al., 2008).

The role of Rab14 in recycling of cargo from endosomes is a matter of debate. Two studies have shown no effect of Rab14 on recycling by overexpressing a dominant negative and a constitutively active mutant in HeLa and MDCK cells (Junutula et al., 2004; Kitt et al., 2008). However, Rab14 has been shown to co-localize on endosomes with Rab4 and Rab11 and also to interact with the Rab4 effector Rabip4 and the Rab11 effector FIP2, RCP and Rip11 (Kelly et al., 2010; Yamamoto et al., 2010). Furthermore, knockdown of Rab14 slowed recycling of the TfR from endosomes in HeLa cells (Yamamoto et al., 2010).

Next to sorting at the TGN and endosomal recycling, a role for Rab14 in the degradative pathway has also been suggested. It was shown that overexpression of dominant negative Rab14 accelerated EGF degradation (Proikas-Cezanne et al., 2006).

Recycling from and Carrier Formation at Endosomes

Recycling from endosomes is complex, since it involves several different pathways. Cargo molecules recycle from early sorting or recycling endosomes. Additionally, cargo molecules may be recycled from the same type of endosomes by differing mechanisms. It is likely that more than one type of transport carrier is used, similarly to the various mechanisms of endocytosis (Grant and Donaldson, 2009).

To investigate the process of recycling *in vivo,* a cargo protein has to be chemically, fluorescently or radioactively labeled at the cell surface, so it can be tracked. Following endocytosis, the intracellular cargo molecule is then allowed to recycle to the plasma membrane, while re-endocytosis has to be prevented. Cellular disappearance or extracellular appearance of the cargo molecule is then measured and compared in wild-type cells and cells overexpressing a protein or mutant protein, or cells in which gene expression was silenced. The TfR offers the advantage that its ligand Tf is the only ligand known to efficiently recycle, which makes it easier to label it and prevent re-endocytosis.

For example, Rab4 has been studied with such recycling assays, however, with varying results. For instance, knockdown of Rab4 in HeLa cells led to an increased recycling rate of Tf in one study and a decreased recycling rate in another (Deneka et al., 2003; Yamamoto et al., 2010). These studies have, however, both looked at relatively few and broadly spaced time points that were at least 10 min apart. It is therefore relevant to investigate the role of Rab4 in recycling more closely, for example by the use of a more quantitative recycling assay, in which many thousand cells are measured per time point, and with improved time resolution.

On the other hand, *in vivo* experiments have the drawback of possible indirect effects, because expression or knockdown of proteins takes many hours to days to take effect. Meanwhile compensatory mechanisms could take over, a possibility especially relevant for the recycling system that consists of more than one pathway. Additionally, *in vivo* experiments like a recycling assay often observe complete pathways rather than specific steps. Therefore it makes sense to complement data of *in vivo* and *in vitro* experiments.

Our lab has developed an *in vitro* assay to specifically monitor the step of vesicle formation at endosomes in semi-intact cells (Pagano et al., 2004). This *in vitro* transport carrier formation assay is

used to reconstitute carrier formation at endosomes under defined conditions using semi-intact cells, exogenous cytosol and an energy source. In a first step proteins are biotinylated at the cell surface. The cells are then allowed to endocytose the biotinylated proteins resulting in endomembranes loaded with biotinylated proteins. Subsequently the remaining biotin at the outside of the cell is stripped from the plasma membrane. The cells are then perforated and endogenous cytosol is removed. Cytosol, energy, and an energy regenerating system are added and carrier formation from any compartment is allowed *in vitro.* Membranous carriers are separated from donor compartments by centrifugation, and proteins are isolated with avidin beads and visualized by immunoblotting.

Cytosol modification, for example by addition of proteins, mutant proteins, chemicals or depletion of proteins can be used to study the influence of the protein of interest on carrier formation. However, very specific antibodies are needed for efficient depletion. Another method is to purify the protein of interest, which can be difficult and time consuming. To circumvent antibody and purification difficulties, another possibility to manipulate the cytosol is to express putative candidates involved in the formation of transport carriers or mutant proteins in cultured cell lines and to extract the cytosol. The use of dominant mutations is necessary to overcome the activity of the endogenous protein that is still present in the cytosol. Furthermore, knockdown cell lines can be used to produce cytosol depleted of a candidate protein.

Aim of this Thesis

Rab proteins play an important role in many transport steps throughout the secretory system. While the mechanisms and pathways of endocytic recycling are not entirely characterized, it is known that Rabs are involved. However, the precise function of the Rabs is often not altogether clear, as the conflicting reports on the function of Rab4 illustrate. It is therefore interesting to investigate Rab4 and other Rabs implicated in endocytic recycling more closely. Furthermore, elucidation of the role of Rab proteins will help the understanding of the regulation of intracellular membrane traffic.

The first goal of this work was to establish a system to produce cytosol from cultured cells in sufficient quantity and quality to be used in the *in vitro* transport carrier formation assay. Cytosol produced from cells expressing Rab4, Rab11 and Rab14 or their mutants was then used in the *in vitro* assay to investigate the role of these Rabs on endosomal carrier formation. The second goal of this work was to establish a recycling and endocytosis assay using fluorescently labeled transferrin as a ligand, and automated microscopy and image analysis to quantify the data. This assay was then used to investigate the effects of dominant mutants or RNAi mediated silencing of Rab4 and Rab11 on recycling.

Materials and Methods

Materials

Reagents

Deferoxamine, bovine holo-transferrin, monensin, benzamidine, pepstatin A, leupeptin, antipain and chymostatin were from Sigma. Dynasore was from Ascent Scientific. Adenosine-5'-triphosphate, guanosine-5'-triphosphate, creatine phosphate, creatine kinase were all from Roche Diagnostics. The Bradford protein assay and the Precision Plus (All Blue Standards) molecular marker were from Bio-Rad. Biotin conjugated transferrin, Alexa-Fluor conjugated transferrin and Alexa-Fluor conjugated phalloidin were from Invitrogen. DY-647-phalloidin was from Dyomics. HRP conjugated streptavidin was from Thermo Scientific. Avidin-Sepharose was from Pierce Chemical. Protein A-Sepharose was from Zymed Laboratories. Protein G-Sepharose was from Gerbu Biotechnik.

Antibodies

Rabbit anti-Rab4 antibody was a gift from Bruno Goud (Institut Curie, Paris, France). Rabbit anti-Rab11 antibodies were from Zymed/Invitrogen and Cell Signaling Technology. Rabbit anti-Rab7 antibody was from Cell Signaling Technology. Rabbit anti-Rab14 antibody was from Aviva Systems Biology. Mouse anti-Rab15 antibody was from Lifespan Biosciences. Mouse anti-TfR antibody was from Invitrogen. Mouse anti-GFP antibody was from Roche Applied Science. Mouse anti-Tubulin was a gift from Hesso Farhan (Biotechnology Institute Thurgau at the University of Constance, Kreuzlingen, Switzerland). Mouse anti-AP1 (γ-adaptin) antibody was from Alexis biochemicals. Mouse anti-Rabaptin-5 was from BD Bioscience. Mouse 2g2 IgM anti-actin antibody was a gift from Cora-Ann Schönenberger (Biozentrum of the University of Basel, Basel, Switzerland). The secondary, horseradish peroxidase coupled antibodies goat anti-rabbit IgG and goat anti-mouse IgG as well as the FITC-conjugated goat anti-mouse IgM antibody were from Sigma. The secondary Alexa-Fluor-680 coupled goat anti-mouse IgG antibody was from Molecular Probes.

Methods

Cloning Procedures

Rab4 wild-type, S22N and N121I in the pcDNA3.1 vector were a gift from Peter van der Sluijs (University of Utrecht, Utrecht, The Netherlands), Rab11 wild-type, S25N and Q70L in the pEGFP-C1 vector were a gift from Sven van Ijzendoorn (University of Groningen, Groningen, The Netherlands) and Rab14 wild-type and S25N in the pEGFP-C1 vector were a gift from Tassula Proikas-Cezanne (Eberhard Karls University, Tübingen, Germany).

Rab4 and mutants were amplified from the pcDNA3.1 vector with the rab4bam-s (5`-CGC GGA TCC ACC ATG TCC GAA ACC TAC GAT T-3`) and rab4cla-a (5`-GCG ATC GAT CTA ACA ACC ACA CTC CTG-3`) primers. The PCR products were then ligated into the pTRE2hyg vector via the newly introduced BamHI and ClaI sites. Rab4 and mutants were then cut out of pTRE2hyg with BamHI and SalI and ligated into pcDNA5/TO previously cut with BamHI and XhoI. To introduce the Q67L mutation into Rab4 the adenines at position 198 and 200 were both changed to thymine, which also introduced an XbaI site. Two separate PCR reaction were performed on Rab wild-type in pTRE2hyg, one with the primers rab4bam-s and rab4Q67Lmut-a (5'-CTC GTC ACG GAC CTG AAT CGT TCT AGA CCT GCT G-3') and the other one with the primers rab4cla-a and rab4Q67Lmut-s (5'-CAA ATA TGG GAT ACA GCA GGT CTA GAA CGA TTC-3'). The two resulting PCR products were mixed and a second PCR in the presence of the primers rab4bam-s and rab4cla-a was performed. The resulting full length Rab4 Q67L sequence was the ligated into the pTRE2hyg vector via the BamHI and ClaI sites. From pTRE2hyg it was cut with BamHI and SalI and ligated into pcDNA5/TO via the BamHI and XhoI sites.

Rab11 and mutants were amplified from the pEGFP-C1 vector with the rab11bam-s (5'-CGC GGA TCC ACC ATG GGC ACC CGC GAC GAC-3') and rab11cla-a (5'-GCG ATC GAT TTA TAT GTT CTG ACA GCA CTGC-3') primers. The PCR products were then ligated into the pTRE2hyg vector via the newly introduced BamHI and ClaI sites. Rab11 was then cut out of pTRE2hyg with BamHI and SalI and ligated into pcDNA5/TO previously cut with BamHI and XhoI.

Rab14 and mutants were amplified from the pEGFP-C1 vector with the rab14bam-s (5'-CGCGGATCCACCATGGCAACTACACCGTAC-3') and rab14cla-a (5'-GCG ATC GAT CTA GCA GCC ACA GCC TT-3') primers. The PCR products were then ligated into the pTRE2hyg vector via the newly introduced BamHI and ClaI sites. Rab11 was then cut out of pTRE2hyg with BamHI and SalI and ligated into pcDNA5/TO previously cut with BamHI and XhoI. To introduce the Q70L mutation into Rab14 the adenines at position 207 and 209 were both changed to thymine and the guanine at

position 210 was changed to adenine, which also introduced an XbaI site. Two separate PCR reaction were performed on Rab wild-type in pTRE2hyg, one with the primers rab14bam-s and rab14Q70Lmut-a (5'-GTA ACA GCC CTA AAT CGC TCT AGA CCT GCC GTA TCC-3') and the other one with the primers rab14cla-a and rab14Q70Lmut-s (5'-CAG ATT TGG GAT ACG GCA GGT CTA GAG CGA TTT AGG-3'). The two resulting PCR products were mixed and a second PCR in the presence of the primers rab14bam-s and rab14cla-a was performed. The resulting full length Rab14 Q70L sequence was then liagted into the pTRE2hyg vector via the BamHI and ClaI sites. From pTRE2hyg it was cut with BamHI and SalI and ligated into pcDNA5/TO via the BamHI and XhoI sites.

To generate Rab4 and Rab11 knockdown cell lines, the pSUPERIOR.neo+GFP vector was used, that contained a human H1 polymerase-III promoter for shRNA expression. To silence Rab4, the sequence 5'-AGG TCC GTG ACG AGA AGT TAT T-3', which corresponds to nucleotides 433-454, was chosen. The oligos Rab4a433-f (5'-GAT CCC GGT CCG TGA CGA GAG TTA TTC AAG AGA TAC CTT CTC GTC ACG GAC CTT TTT GGG A-3') and Rab4a433-r (5'-AGC TTT TCC AAA AAG GTC CGT GAC GCG AAG TTA TCT CTT GAA TAA CTT CTC GTC ACG GAC CGG-3'), containing the sense and antisense target sequence, a hairpin loop, and a BglII and HindIII site were annealed, and then ligated into pSUPERIOR.neo+GFP via the BglII and HindIII sites. To silence Rab11, the sequence 5'-ACG ACG AGT ACG ACT ACC TAT T-3', which corresponds to nucleotides 18-39, was chosen. The oligos Rab11b18-f (5'-GAT CCC CCG ACG AGT ACG ACT ACC TAT TCA AGA GAT AGG TAG TCG TAC TCG TCG TTT TTG GAA-3') and Rab11b18-r (5'-AGC TTT TCC AAA AAC GAC GAG TAC GAC TAC CTA TCT CTT GAA TAG GTA GTC GTA CTC GTC GGG-3') containing the sense and antisense target sequence, a hairpin loop, and a BglII and HindIII site were annealed and then ligated into pSUPERIOR.neo+GFP via the BglII and HindIII sites.

Cell Cultures and Transfections

Cos-1 cells were cultured in Dulbecco's Modified Eagle's Medium – low glucose (Sigma) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum at 37°C with 7.5% CO_2. Cos-1 cells were transfected using Lipofectin (Invitrogen).

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium – high glucose (Sigma) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum at 37°C with 7.5% CO_2. HeLa cells were transfected using FuGENE HD (Promega) for plasmid DNA and Lipofectamine RNAiMAX (Invitrogen) for siRNA (ON-TARGETplus SMARTpool siRNA, Dharmacon).

HeLa T-REx cells (Invitrogen) were cultured in Minimum Essential Medium Eagle (Sigma) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml blasticidin and 10% fetal calf serum at 37°C with 7.5% CO_2. HeLa T-REx cells were transfected using Lipofectin (Invitrogen). To generate stable cell lines expressing the Rab constructs, clones resistant to 180 µg/ml hygromycin were isolated and expression was assessed by immunoblot analysis. To generate stable cell lines silencing Rab4 and Rab11 by shRNA expression, clones resistant to 1 mg/ml G418-sulphate were isolated and expression was assessed by immunoblot analysis.

BHK cells were cultured in Glasgow Minimum Essential Medium (Sigma) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 5% fetal calf serum and 10% tryptose phosphate broth at 37°C with 5% CO_2.

SDS-PAGE and Immunoblotting

For immunoblot analysis samples were run on 12.5% or 15% acrylamide containing SDS-PAGE gels to separate the proteins. Proteins were transferred for 1 h at 48 volts on to an Immobilon-P^{SQ} membrane (Millipore). The membrane was blocked 1 h in PBS containing 0.1 mM Tween-20 and 5% w/v nonfat dry milk. The membrane was washed three times 5 min in PBS containing 0.1 mM Tween-20. The membrane was incubated with the primary antibody for 2 h at room temperate or over night at 4°C in PBS containing 0.1 mM Tween-20 and 5% nonfat dry milk or 5% BSA. The membrane was washed four times 5 min in PBS containing 0.1 mM Tween-20. The membrane was incubated with the secondary antibody for 1 h at room temperate in PBS containing 0.1 mM Tween-20 and 5% w/v nonfat dry milk. The membrane was washed three times 5 min in PBS containing 0.1 mM Tween-20 and two times 5 min in PBS. For protein detection Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used and membranes were exposed to Kodak Biomax XAR films or imaged with an ImageQuant LAS 4000 (GE Healthcare). Alternatively, the membrane was scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences) for protein detection. Band intensities were quantified using the ImageJ software.

Indirect Immunofluorescence Microscopy

Cells were washed three times with PBS and fixed for 10 min with 3% paraformaldehyde in PBS. Cells were then washed three times with PBS, incubated 5 min with 50 mM NH_4Cl in PBS and washed again three times with PBS. The cells were then permeabilized with 0.1% (w/v) Triton-X-100 in PBS for 5 min, washed three times with PBS and incubated 15 min with 1% (w/v) BSA in PBS. The cells were then incubated for 1 to 2 h or overnight at 4 °C in a humidified chamber in 1% (w/v) BSA in PBS

containing the primary antibody at appropriate concentration. They were then washed twice with 1% (w/v) BSA in PBS and twice with PBS for 5 min each before they were incubated for 30 min in 1% (w/v) BSA in PBS containing the appropriate fluorescently labeled antibody at appropriate concentration, 10 µM Bisbenzimide H33342 (Hoechst stain) and, in some cases, 1 unit/ml Alexa-fluor labeled phalloidin. Cover slips were mounted in mowiol and allowed to set for at least 30 min. The samples were analyzed with a Zeiss Axioplan 2 immunofluorescence microscope with a Leica DFC420 C imaging system and images were processed by Adobe Photoshop.

Cytosol Production from Cultures Cells

Cytosol from HeLa T-REx cells was harvested 24 h post induction (1 µg/ml doxycycline) and from Cos-1 cells 24 h post transfection. Cells were washed twice with PBS and incubated with 0.5 mM EDTA at 37°C for 15 min to detach the cells. To collect the cells they were centrifuged for 5 min at 800 × g. The supernatant was removed and the cells were resuspended in an equal volume of ice cold transport buffer (20mM HEPES/KOH, pH 7.2, 90 mM KOAc, 2 mM $Mg(OAc)_2$) supplemented with 1 mM PMSF and 1 × protease inhibitor cocktail (PIC) from a 500 × stock (5 mg/ml benzamidine, 1 mg/ml pepstatin A, leupeptin, antipain, chymostatin). The cells were then homogenized with 15 strokes of a motorized glass-Teflon homogenizer. The homogenate was centrifuged for 30 min at 8300 × g at 4°C. The supernatant was centrifuged for 1 h at 130000 × g at 4°C. The supernatant, representing cytosol, was snap frozen in liquid nitrogen and stored at -80°C. Protein concentration was determined using the Bradford protein assay (Bio-Rad).

Immunoprecipitation

For immunodepletion, a 1:1 mixture of protein A- and protein G-Sepharose beads were incubated with 1 µg/µl of the anti-AP1 (γ-adaptin) antibody in PBS containing 50 µM Tris, pH8 over night at 4°C in an end-over rotator. The beads were then washed twice with PBS and 40 µl of the packed beads were incubated with 100 µl of calf brain cytosol for 2 h or 4 h at 4°C in an end-over rotator. 10 µl of the depleted cytosol were then separated by SDS-PAGE and the presence of AP-1 was assessed by immunoblotting. As a control, cytosol was mock treated with beads without antibody.

In Vitro Endosomal Transport Carrier Formation Assay

BHK cells were washed three times with ice cold PBS. They were then incubated with 1 mg/ml Sulfo-NHS-SS-Biotin (Thermo Scientific) in PBS for 30 min on a rocker. The cells were washed twice with ice cold PBS and the Sulfo-NHS-SS-Biotin was quenched with 50 mM glycine in PBS for 5 min. The cells were rinsed twice with ice cold PBS and then incubated at 37°C for 10 min with prewarmed medium supplemented with 20 mM HEPES. The cells were rinsed twice with ice cold PBS and then incubated twice 20 min with glutathione solution (50 mM reduced gluthathione, 75 mM NaCl, 75 mM NaOH, 1 mM EDTA and 1% (w/v) BSA). After two washes with ice cold PBS the cells were incubated for 5 min with 0.5% iodoacetamide in PBS. The cells were then washed twice with ice cold PBS and incubated with swelling buffer (15 mM HEPES/KOH, pH 7.2, 15 mM KCl) for 15 min at 4°C. The swelling buffer was replaced with transport buffer (20 mM HEPES/KOH, pH 7.2, 2 mM $Mg(OAc)_2$, 90 mM KOAc) and the cells were scraped of the dish with a rubber policeman. The scraped solution was centrifuged at 800 × g for 5 min at 4°C. The supernatant was discarded, the pellet resuspended in high salt buffer (20 mM HEPES/KOH, pH 7.2, 500 mM KOAc, 2 mM $Mg(OAc)_2$), incubated on ice for 10 min and then centrifuged at 800 × g for 5 min at 4°C. This step was repeated once. The pellet was then resuspended in 400 µl transport buffer. For every condition of the assay 100 µl of the cell resuspension was added to 10 µl 10 × transport buffer, 20 µl energy mix (100 mM ATP, 200 mM GTP, 600 mM creatine phosphate and 8 mg/ml creatine kinase) and varying volumes of cytosol and ddH_2O to a total volume of 200 µl. Every condition was incubated for 30 min at 37°C. The assay mixes were then centrifuged at 800 × g for 5 min at 4°C. The pellet was kept and the supernatant was centrifuged at 20000 × g for 5 min at 4°C. The supernatant and the pellet of the first centrifugation step were then lysed in lysis buffer (1% Triton-X-100 and 0.5% deoxycholate in PBS) supplemented with 1 mM PMSF and 1 × PIC for 1 h at 4°C. After this 30 µl of pre-equilibrated avidin-Sepharose beads (Pierce) were added and the mix was incubated with end over rotation for 1 h at 4°C. The beads were then washed three times with lysis buffer and carrier formation was assessed by immunoblot analysis.

In Vivo Endocytosis and Recycling Assays

To assess endocytosis, cells were starved for 2 h in medium supplemented with 20 mM HEPES. The cells were then washed once with medium supplemented with 20 mM HEPES and incubated at 37°C with medium supplemented with 20 mM HEPES and 50 µg/ml Alexa-Fluor tagged transferrin for the desired time. The cells were then quickly cooled to 4°C and washed twice with stripping buffer (150 mM NaCl, 50 mM NaAc, pH 3.5). After this the cells were washed four times with ice cold PBS and fixed with 3% paraformaldehyde in PBS. Then the cells were washed three times with PBS, incubated

with 50mM NH_4Cl in PBS for 5 min and washed three times with PBS, again. The cells were then incubated with 0.1% Trition-X-100 in PBS for 5 min, washed four times with PBS and incubated with 1 unit/ml Alexa-Fluor tagged phalloidin and 10 µM Bisbenzimide H33342 (Hoechst dye) in PBS for 30 min. After that the cells were washed four times with PBS.

To assess recycling, cells were starved for 2 h in uptake medium (medium supplemented with 20 mM HEPES). The cells were then washed once with uptake medium incubated for 1 h at 37°C with uptake medium supplemented with 50 µg/ml Alexa-Fluor tagged transferrin. Afterwards the cells were cooled to 4°C by washing them three times with cold PBS. They were then washed twice with stripping buffer (150 mM NaCl, 50 mM NaAc, pH 3.5) and again four times with ice cold PBS. The cells were then quickly warmed to 37°C and transferrin was chased for appropriate times in the presence of uptake medium supplemented with 50 mM deferoxamine mesylate salt (Sigma). To stop the chase the cells were then fixed with 3% paraformaldehyde in PBS. Then the cells were washed three times with PBS, incubated with 50mM NH_4Cl in PBS for 5 min and washed three times with PBS, again. The cells were then incubated with 0.1% Trition-X-100 in PBS for 5 min, washed four times with PBS and incubated with 1 unit/ml Alexa-Fluor tagged phalloidin and 10 µM Bisbenzimide H33342 (Hoechst dye) in PBS for 30 min. After that the cells were washed four times with PBS.

To assess recycling in HeLa T-REx cells, the cells were starved for 2 h in uptake medium (medium supplemented with 20 mM HEPES). The cells were then washed once with uptake medium incubated for 1 h at 37°C with uptake medium supplemented with 50 µg/ml Alexa-Fluor tagged transferrin. The cells were then washed once with PBS, and transferrin was chased for appropriate times in the presence of uptake medium supplemented with 50 mM deferoxamine mesylate salt (Sigma). To stop the chase the cells were then cooled to 4°C and washed twice with ice cold PBS. They were then fixed with 3% paraformaldehyde in PBS. Then the cells were washed three times with PBS, incubated with 50mM NH_4Cl in PBS for 5 min and washed three times with PBS, again. The cells were then incubated with 0.1% Trition-X-100 in PBS for 5 min, washed four times with PBS and incubated with 1 unit/ml Alexa-Fluor tagged phalloidin and 10 µM Bisbenzimide H33342 (Hoechst dye) in PBS for 30 min. After that the cells were washed four times with PBS.

Image acquisition was done automatically with an ImageXpress Micro (Molecular Devices), and image analysis was performed with the CellProfiler software (Carpenter et al., 2006).

Results

Part I: *In Vitro* Formation of Transport Carriers

Our lab has established an *in vitro* assay to study the formation of transport carriers at endosomes. In contrast to *in vivo* studies, where often a complete pathway is observed, this assay can be used to investigate the role of candidate proteins on the specific step of carrier formation. In the assay, transport carrier formation is reconstituted under defined conditions, using semi-intact cells, cytosol and an energy source. The influence of different factors on carrier formation can then be studied by manipulating the cytosol. In a previous study of our lab, MDCK cells and calf brain cytosol were used for this assay (Pagano et al., 2004). Even though MDCK cells are polarized, it is highly likely that only non-polarized recycling was reconstituted, since calf brain cytosol only contains the necessary machinery for non-polarized recycling. Nevertheless, we chose to switch to non-polarized cells. Furthermore, instead of calf brain cytosol, cytosol derived from cultured cells was used. The advantage of cultured cells is that it is possible to manipulate the cells, and thus the cytosol before it is prepared, expanding the range of manipulations beyond what is possible with calf brain derived cytosol.

Cytosol Preparation from Cultured Cells

As described above, our lab has established an *in vitro* endosomal transport carrier formation assay to specifically monitor the step of vesicle formation at endosomes in semi-intact cells (Pagano et al., 2004). In order to study the importance of a protein in carrier formation, the cytosol used in the assay needs to be modified. Possibilities to modify the cytosol include immunodepletion or immunoinhibition of the protein of interest, or the addition of purified proteins or mutant proteins, or chemicals like non-hydrolysable nucleotides. However, immunodepletion or immunoinhibition is not always possible due to the lack of suitable antibodies. Furthermore, protein purification can be difficult and time consuming, and many mammalian proteins like Rabs, which are prenylated, are not easily made in bacterial expression systems (e.g. E.coli). They need to be produced in a eukaryotic expression system (e.g. insect cells), because bacteria lack many of the post translational modifications of eukaryotes. To circumvent these difficulties we wanted to develop a system to prepare cytosol from cultured cell lines. The advantage of this system is that proteins of interest or dominant mutants thereof can be expressed in mammalian cells, and subsequently the cytosol from

these cells can be extracted and used in the endosomal transport carrier formation assay. Furthermore, cytosol depleted of a protein of interest can be obtained by using RNA interference to silence its protein expression.

To determine whether it was possible to produce sufficient amounts of cytosol in feasible concentrations, cytosol was prepared from Cos-1 and HeLa T-REx (Invitrogen) cells. Cos-1 cells were chosen because they have the ability to amplify introduced plasmids, which makes them useful to produce high amounts of proteins in transiently transfected cells. HeLa T-REx cells were chosen because they make use of a tet-ON system that can be employed for regulated expression of proteins in stably transfected cells (Yao and Eriksson, 1999; Yao et al., 1998).

The procedure to produce cytosol from cultured cells was adapted from a previously established protocol to purify clathrin coated vesicles from HeLa cells (Hirst et al., 2004). Briefly, confluent cells were detached from the tissue culture dish with 0.5 mM EDTA. The cells were then collected by low speed centrifugation and resuspended in an equal volume of ice cold transport buffer containing protease inhibitors. They were then homogenized and the homogenate was first centrifuged at low speed to pellet the heavy membranes. This post-nuclear supernatant was then centrifuged at high speed to pellet all remaining membranes. The total volume and protein concentration of the cytosol were measured, and the total protein amount calculated.

For every condition in the endosomal carrier formation assay, 200 µg of total protein is added. To achieve this, the cytosol needs a concentration of at least 3 µg/µl. The concentration of the prepared cytosol ranged from approximately 4 µg/µl to 16 µg/µl for HeLa T-REx cells and 6 µg/µl to 24 µg/µl for Cos-1 cells. The average protein concentration was 9 µg/µl for HeLa T-REx and 14 µg/µl for Cos-1 cells (Figure 9A). The total protein amount ranged from approximately 1.5 mg to 9.5 mg for HeLa T-REx and 6 mg to 16 mg for Cos-1 cells. The average total protein amount was 3.6 mg for HeLa T-REx and 10.8 mg for Cos-1 cells (Figure 9B). The fluctuations in protein concentration can be explained by the difficulty to resuspend the cells in an equal amount of transport buffer after they have been harvested. This leads to variations in the dilution of the cytosol with transport buffer. The fluctuation in total protein isolated can be explained by differences in the amount of grown cells and by losses during harvesting, resuspension, and homogenization. The differences between HeLa T-REx cells and Cos-1 cells can be explained by differences in cell morphology and the fact that HeLa T-REx cells do not grow to full confluency.

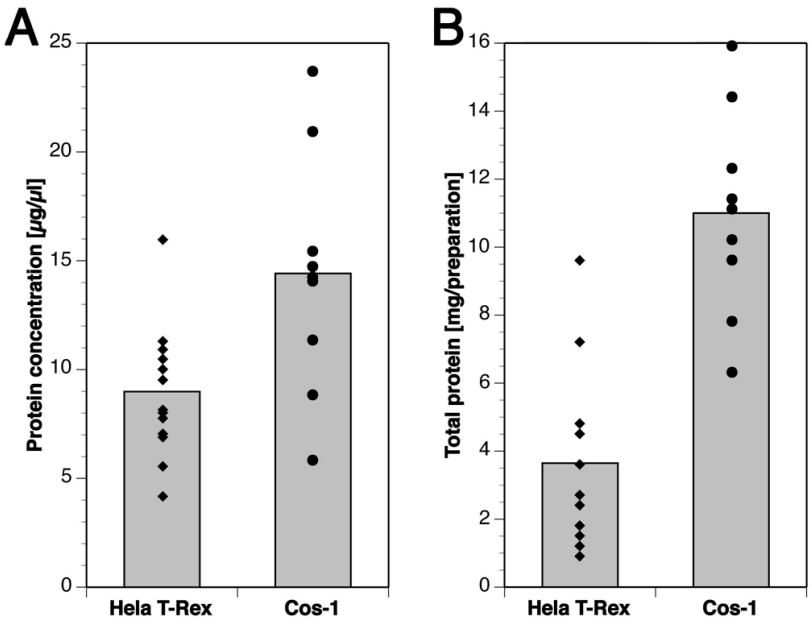

Figure 9. Cytosol can be produced in sufficient quantity and concentration from Cos-1 and HeLa T-REx cells.

(A) Cytosol was produced from Cos-1 and HeLa T-REx cells. The grey columns represent the average protein concentration of 13 preparations from HeLa T-REx and 9 from Cos-1 cells. The dots represent the protein concentration of individual cytosol preparations.
(B) The grey columns represent the average total protein amount of 13 preparations from HeLa T-REx and 9 from Cos-1 cells. The dots represent the the total protein amount of individual cytosol preparations

Cytosol from HeLa T-REx and Cos-1 Cells Promotes Endosomal Transport Carrier Formation

Next we wanted to know whether cytosol prepared from HeLa T-REx and Cos-1 cells promotes carrier formation at endosomes to a similar extent as calf brain cytosol. The endosomal transport carrier formation assay was performed in previously described BHK cells stably expressing the EGF receptor coupled to GFP (green fluorescent protein) (Petiot et al., 2003). The use of this cell line makes it possible to track formation of carriers containing the TfR, a typical constiutively recycling transport receptor, and the EGFR, a ligand-dependent signaling receptor. Unbound EGFR is efficiently recycled to the plasma membrane upon endocytosis, while EGFR activated by EGF is sorted towards the lysosomes and degraded, making it possible to observe two distinct sorting steps at early sorting endosomes (Roepstorff et al., 2008).

The *in vitro* carrier formation assay has been established in polarized MDCK cells, therefore we first checked whether the assay was also feasible in the non-polarized BHK cells (Pagano et al., 2004). To test this, we determined whether carrier formation was depended on the presence of cytosol and AP-1, since AP-1 depletion had a prominent inhibiting effect on carrier formation in the MDCK cells.

Therefore, calf brain cytosol was depleted of AP-1 by using an anti-γ-adaptin antibody (Figure 10A).To test the effect of cytosol and AP-1 depleted cytosol on carrier formation, intact BHK cells were biotinylated at 4°C with sulfo-NHS-SS-biotin. The cells were then incubated at 37°C for 10 min with medium containing HEPES to allow endocytosis of biotinylated surface receptors. The cells were then cooled again to 4°C and surface biotin was removed by washing with a reduced glutathione solution, while intracellular biotinylated proteins were protected. Permabilization of the cells was achieved by swelling and scraping them. To remove cytosol, proteins peripherally associated with the cytosolic face of membranes, and free transport vesicles, the broken cells were washed twice with high-salt buffer. These broken cells were then incubated with ATP, GTP, an energy regenerating system, and with untreated calf brain cytosol, AP-1 depleted cytosol, mock depleted cytosol, or no cytosol at 37°C for 30 min. Vesicles released during this incubation period were recovered in the supernatant after two consecutive centrifugation steps at 800 × g and 20'000 × g. The vesicles were then lysed, and biotinylated proteins were isolated using avidin-Sepharose. EGFR was detected by SDS gel electrophoresis and immunoblotting. Carrier formation was strongly dependent on the presence of cytosol (Figure 10B and 10C), and depletion of AP-1 clearly inhibited carrier formation in comparison to the use of untreated cytosol or mock-depleted cytosol (Figure 10B).

To test whether Cos-1 and HeLa T-REx cytosol are able to promote *in vitro* carrier formation, the assay was perfomed as described above. The broken cells were incubated with ATP, GTP, an energy regenerating system, and with or without cytosol from calf brain, Cos-1 cells, or HeLa T-REx cells at 37°C for 30 min. Recovered vesicles were lysed, and biotinylated proteins were isolated using avidin-Sepharose. TfR and EGFR were detected by SDS gel electrophoresis and immunoblotting. As is shown in Figure 10C, cytosol derived from calf brain, Cos-1 cells, and HeLa T-REx cells significantly stimulated carrier formation at endosomes in comparison to incubation without cytosol.

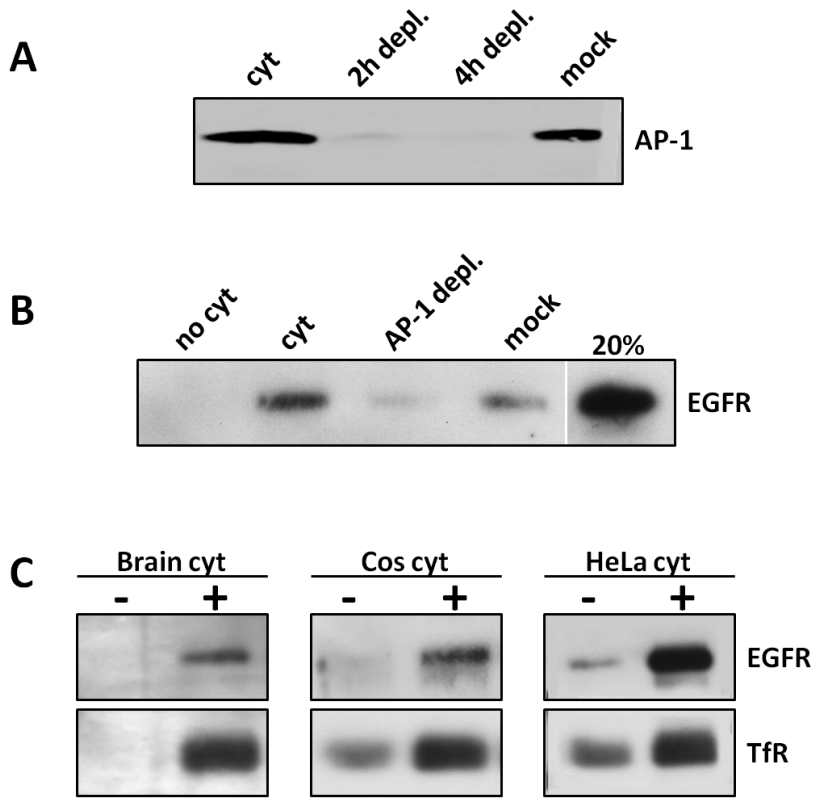

Figure 10. *In vitro* endosomal carrier formation in BHK cells is AP-1 dependent and is promoted by cell culture cytosol

(A) Calf brain cytosol was immunodepleted of AP-1 by using anti-γ-adaptin antibody and protein A/G-Sepharose beads. Untreated cytosol (cyt) and depleted cytosol (2h depl. And 4h depl.) were analyzed by immunoblotting.
(B) Biotinylated permeabilized BHK cells were incubated in the presence of ATP, GTP, and ATP-regenerating system without cytosol (no cyt) with normal cytosol (cyt), with cytosol depleted of AP-1 (AP-1 depl.) or with mock depleted cytosol (mock). Immunoblot analysis of biotinylated EGFR in the supernatant after cell pelleting is shown. 20% of the total biotinylated material is shown as a control.
(C) Cytosol derived from calf brain, Cos-1 cells and HeLa T-REx cells was used in the endosomal carrier formation assay. Biotinylated permeabilized BHK cells were incubated in the presence of ATP, GTP, and ATP-regenerating system with or without cytosol. Immunoblot analysis of biotinylated EGFR and TfR in the supernatant after cell pelleting is shown.

Generation of HeLa T-REx Cell Lines Stably Expressing Rab4, Rab11 and Rab14 as well as their Dominant Negative and Active Mutants

In order to be able to produce large quantities of cytosol containing overexpressed or modified proteins, and cytosol depleted of our proteins of interest, stable cell lines were generated. As cell line, HeLa T-REx cells from Invitrogen were chosen, because they make use of a specific tet-ON system. In this system, the introduced plasmid contains two tetO operators that are positioned between the TATA box of the promoter and the transcriptional start site of the protein of interest. The T-REx cells constitutively express TetR, a protein that binds to the tetO sites in the absence of tetracycline, thereby physically blocking the transcription machinery (Yao and Eriksson, 1999; Yao et al., 1998). In the presence of tetracycline or its semisynthetic variant doxycycline, TetR is released from the tetO sites allowing transcription to proceed.

To generate cells stably overexpressing Rab proteins, HeLa T-REx cells were transfected with a plasmid containing either the wild-type, dominant negative, or constitutively active form of either Rab4, Rab11 or Rab14. The dominant negative serine to asparagine mutants are GDP locked, while the asparagine to isoleucine mutant is impaired in guanine nucleotide binding. The constitutively active glutamine to leucine mutants are GTPase defective. Individual clones were selected for their resistance to Hygromycin B. Expression was assessed by SDS gel electrophoresis and immunoblot analysis of cellular lysates from clonal cells induced for 24 h with 1 µg/µl doxycycline or left uninduced. Stable cell lines expressing Rab4 wild-type, dominant negative (S22N, N121I), constitutively active (Q67L), Rab11 wild-type, dominant negative (S25N), constitutively active (Q70L) and Rab14 wild-type, dominant negative (S25N), constitutively active (Q70L) were successfully generated (Figure 11). Overexpression in induced cells was at least 5-fold compared to uninduced cells as determined by semi-quantitative immunoblot analysis.

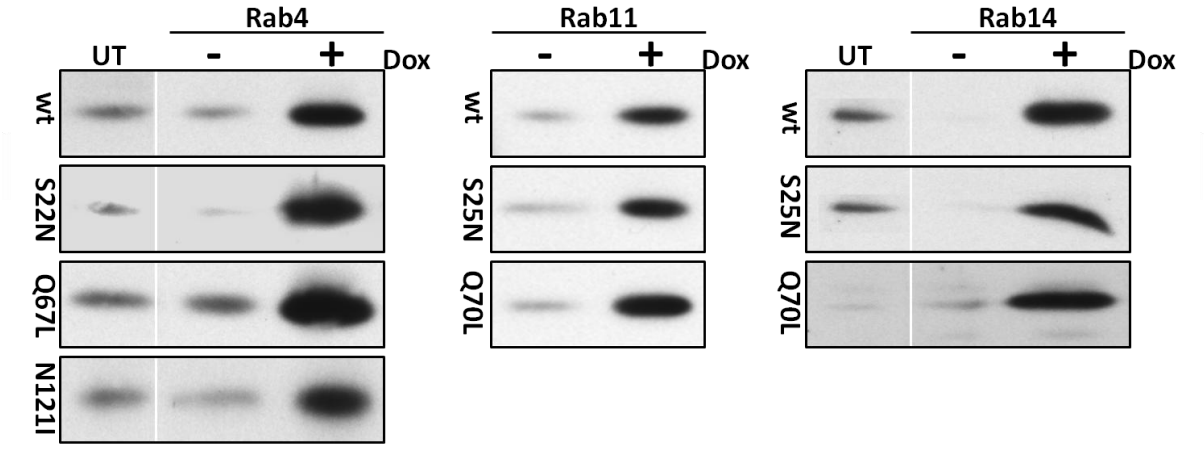

Figure 11. HeLa T-REx cells stably expressing wild-type, dominant negative, and constitutively active Rab4.

HeLa T-REx cells were transfected with plasmids containing wild-type, dominant negative or constitutively active Rab4, Rab11 and Rab14. Resistant clones were either induced 24 h with 1 µg/µl doxycycline (dox) or left uninduced. Total cell lysates were produced and expression levels of induced cells were compared to uninduced cells and untransfected cells (UT) after SDS gel electrophoresis and immunoblotting. Lanes separated by white lines are from the same blot.

We also created stable cell lines expressing shRNA (small hairpin RNA) of Rab4 and Rab11, to silence expression of these two proteins via RNA interference. HeLa T-REx cells were used again in order to be able to induce the transcription of the shRNA. Individual clones were selected for their resistance to Neomycin. Gene silencing was assessed by SDS gel electrophoresis and immunoblot analysis of cellular lysates from clonal cell lines induced for 72 h with 1 µg/µl doxycycline or left uninduced. Stable cell lines in which Rab4 and Rab11 expression was silenced, were successfully generated

(Figure 12). Knock-down was approximately 90% in induced cells compared to uninduced cells as determined by semi-quantitative immunoblot analysis.

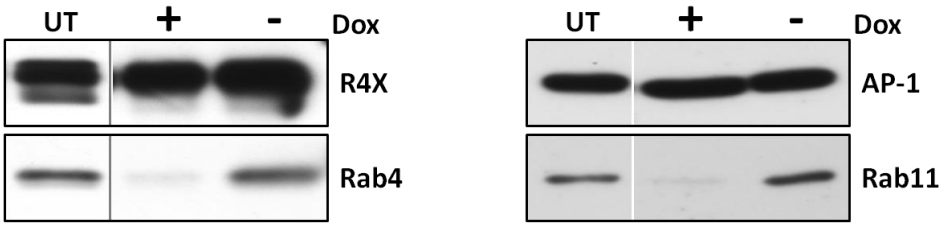

Figure 12. HeLa T-REx cells stably expressing shRNA against Rab4 and Rab11.

HeLa T-REx cells were transfected with a plasmid containing either a shRNA sequence against Rab4 or Rab11. Resistant clones were either induced 72 h with 1 µg/µl doxycycline (dox) or left uninduced. Total cell lysates were produced and knock-down levels of induced cells were compared to uninduced and untransfected cells (UT) after SDS gel electrophoresis and immunoblotting. AP-1, or a cross-reaction band from the Rab4 antibody (R4X), were used as loading controls. Lanes separated by grey or white lines are from the same blot.

Membrane Association of Overexpressed Rab Proteins

Rab proteins are prenylated at their C-terminus which is necessary for membrane association of the protein. For example, Rab11 lacking the prenylation motif does not localize to membranes anymore (Ren et al., 1998). Rabs exert their function when they are membrane bound, and therefore a major fraction of the cellular Rab proteins localize there. For example 75% of endogenous Rab4 is found to associate with the membrane fraction in CHO cells (Van Der Sluijs et al., 1991). It is thus possible that much of the overexpressed Rab proteins associate with the membrane fraction and not the cytosolic fraction during cytosol preparation. This effect could be even more profound in constitutively active mutants, since wild-type Rabs localize to the membrane in their active form. Additionally, the dominant negative Ras mutant acts by sequestering the GEF, thereby inhibiting the activation of cellular Ras (Farnsworth and Feig, 1991; Feig and Cooper, 1988; Powers et al., 1989; Stacey et al., 1991). A similar mechanism has been proposed for Rabs (Li et al., 1994). Since the GEF is potentially membrane associated this could lead to a significant association with the membrane fraction of dominant negative Rabs during cytosol preparation. For example, TRAPP-I is recruited to the COPII coat via Sec23 and once recruited promotes activation of Ypt1p, the yeast Rab1, at the vesicle surface (Sacher et al., 2008).

To address this question Cos-1 cells were transfected with wild-type, dominant negative and constitutively active Rab4. 24 h post transfection cytosol was prepared from the transfected and untransfected cells as described above. 10 µg of total cytosolic proteins were separated by SDS gel

electrophoresis and Rab4 was visualized by immunoblot analysis. As is shown in Figure 13, wild-type, dominant negative, and constitutively active Rab4 was present in Cos-1 cytosol to a similar extent when compared with each other and in an at least 10-fold higher concentration than endogenous Rab4 in untransfected cells, as determined by semi-quantitative immunoblot analysis.

Furthermore, we prepared cytosol from HeLa T-REx cells stably expressing wild-type, dominant negative and constitutively active Rab4 as described above. The pellets of the low speed and high speed centrifugation steps were pooled and then washed with transport buffer. In HeLa T-REx cells expressing wild-type, dominant negative, and constitutively active Rab4 there was a distinct increase in Rab4 concentration in the cytosol as well as slight increase in Rab4 concentration at the membranes (data not shown).

Wild-type and constitutively active Rab proteins interact with effector proteins. Since the Rabs are overexpressed several fold over the endogenous protein in the stably transfected cells, one possibility is that overexpression of wild-type or constitutively active Rabs will recruit a considerable amount of effector proteins to the membrane. In this case the cytosol would contain less effector proteins and any observed effects would not necessarily be solely dependent on the Rab protein itself. To test this, we investigated whether there was a change in the cytosolic and membrane content of Rabaptin-5, an established Rab4 effector (Vitale et al., 1998). The cytosolic fraction and the membrane fraction of HeLa T-REx cells expressing wild-type, dominant negative, and constitutively active Rab4 were compared. Rabaptin-5 was still found to be present in similar quantity in the cytosol of cells expressing high amounts of wild-type, dominant negative and constitutively active Rab4 compared to untransfected cells. Furthermore, no significant increase in membrane associated of Rabaptin-5 was observed in cells expressing any of the Rab mutants compared to untransfected cells (data not shown).

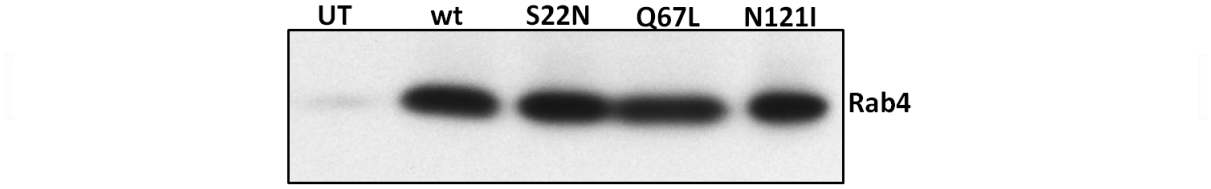

Figure 13. Overexpressed Rab4 is present in the cytosol

Cos-1 cells were transfected with wild-type (wt), dominant negative (S22N, N121I) and constitutively active (Q67L) Rab4 or left untransfected (UT). Subsequently the cytosol was prepared, 10 µg of total protein were separated by SDS gel electrophoresis and Rab4 was detected by immunoblotting.

Rab4 is Involved in the Formation of Transport Carriers at Endosomes

The role of Rab4 in endocytic recycling is not entirely clear. While it has been shown to play a role in recycling, there are inconsistencies regarding the effect of overexpression of a dominant negative mutant or RNAi mediated knockdown (Deneka et al., 2003; McCaffrey et al., 2001; van der Sluijs et al., 1992; Yamamoto et al., 2010). However, Rab4 has been shown to be involved in the *in vitro* formation of transport carriers at endosomes. Overexpression of dominant negative Rab4 in PC12 cells impaired the formation of Tf containing early endosomal vesicles in an *in vitro* assay, and the formation of synaptic-like microvesicles from early endosomes in an *in vivo* assay (de Wit et al., 2001). Furthermore, our lab has shown that depletion of Rab4 from cytosol inhibits the formation of asialoglycoprotein receptor H1 containing endosomal vesicles in MDCK cells (Pagano et al., 2004). We were interested whether Rab4 also played a role in the formation of EGFR and TfR containing endosomal transport carriers in BHK cells. To test this, we performed an endosomal transport carrier formation assay with cytosol from HeLa T-REx cells expressing wild-type, dominant negative or constitutively active Rab4 and from untransfected HeLa T-REx cells. The assay was carried out as described above. As shown in Figure 14 and 15, carrier formation at endosomes was only stimulated in the presence of cytosol. Wild-type Rab4 had no significant effect on the formation of carriers containing TfR and EGFR. Dominant negative (S22N) Rab4 on the other hand had a significant inhibitory effect on carrier formation as shown in Figure 14A and Figure 15B. Interestingly, constitutively active (Q67L) Rab4 had no effect on carrier formation of TfR containing carriers but strongly inhibited the formation of EGFR containing carriers, raising the possibility that TfR and EGFR are transported in different carriers (Figure 14A and Figure 15C). Surprisingly, another dominant negative mutant of Rab4 (N121) had no effect on carrier formation (Figure 14B and 15D). Indeed there is a difference between the two dominant negative mutants, while Rab4 S22N is able to bind GDP but not GTP, Rab4 N121I has a reduced guanine nucleotide binding ability (Cormont et al., 1996; Gerez et al., 2000). It is therefore conceivable that these two mutants do not function in the same way, possibly explaining the differing experimental outcome of the two.

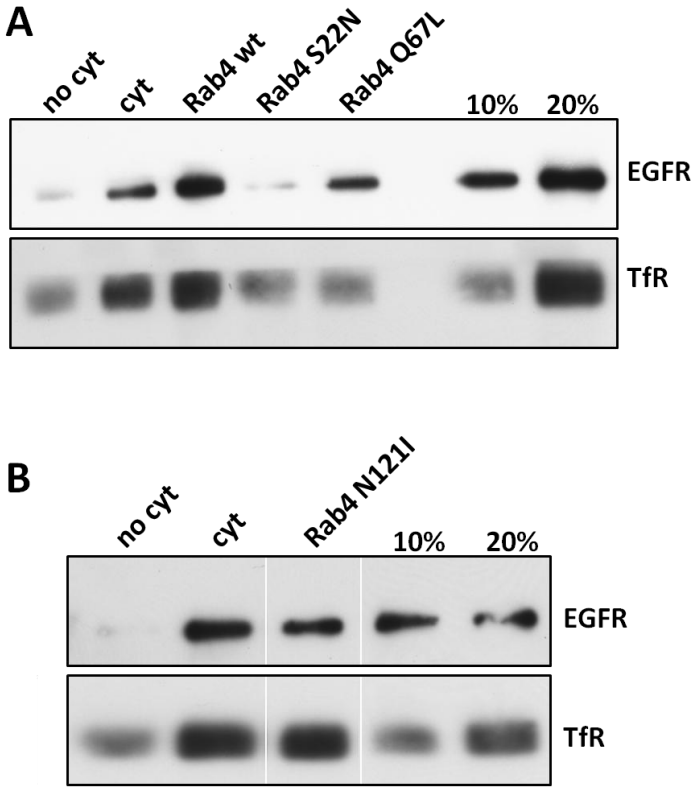

Figure 14. Rab4 is involved in carrier formation at endosomes.

(A) Biotinylated permeabilized BHK cells were incubated in the presence of ATP, GTP, and ATP-regenerating system without cytosol (no cyt), with cytosol (cyt), with cytosol containing wild type Rab4 (Rab4 wt), with cytosol containing dominant negative Rab4 (Rab4 S22N) or with cytosol containing constitutively active Rab4 (Rab4 Q67L). Immunoblot analysis of biotinylated EGFR and TfR in the supernatant after cell pelleting is shown. 10% and 20% of the total biotinylated material are shown as a control.
(B) Biotinylated permeabilized BHK cells were incubated in the presence of ATP, GTP, and ATP-regenerating system without cytosol (no cyt), with cytosol (cyt) or with cytosol containing dominant negative Rab4 (N121I). Immunoblot analysis of biotinylated EGFR and TfR in the supernatant after cell pelleting is shown. Lanes separated by white lines are from the same blot. 10% and 20% of the total biotinylated material are shown as a control.

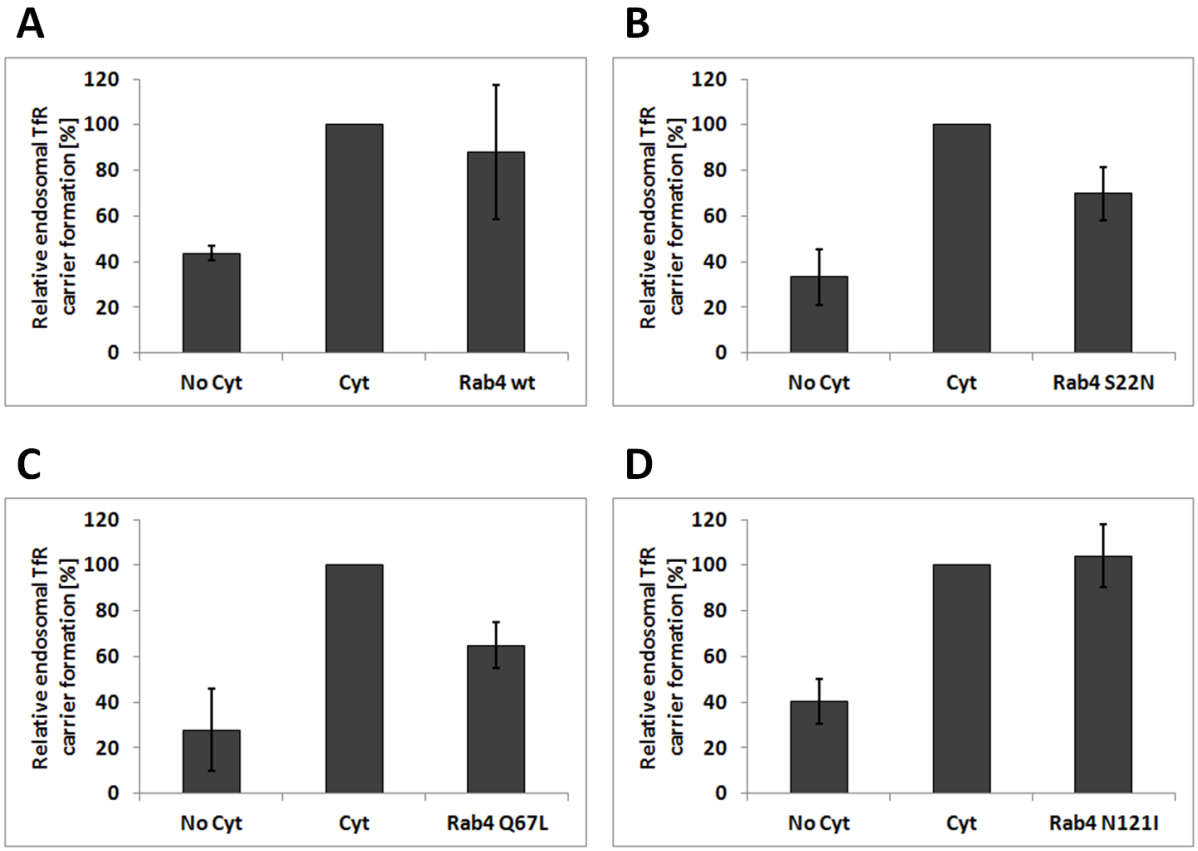

Figure 15. Quantitation of the effect of Rab4 on endosomal TfR carrier formation.

(A) Rab4 wild-type has no significant effect on the formation of endosomal carriers containing TfR. N = 3.
(B) Rab4 S22N has a significant inhibitory effect on the formation of endosomal carriers containing TfR. N = 3.
(C) Rab4 Q67L has a significant inhibitory effect on the formation of endosomal carriers containing TfR. N = 3.
(D) Rab4 N121I has no significant effect on the formation of endosomal carriers containing TfR. N = 4.

Rab11 is Involved in the Formation of Transport Carriers at Endosomes

Rab11 has been shown to affect recycling of the TfR from endosomes to the plasma membrane and was proposed to be involved in the transport of cargo from early sorting to recycling endosomes (Horgan et al., 2010; Ren et al., 1998; Ullrich et al., 1996). However, the precise role of Rab11 is not entirely clear, therefore we wanted to test whether Rab11 is involved in endosomal carrier formation *in vitro*. To test this we performed an endosomal transport carrier formation assay with cytosol from HeLa T-REx cells expressing wild-type, dominant negative or constitutively active Rab11 and from untransfected HeLa T-REx cells. The assay was carried out as described above. As shown in Figure 16 and 17, carrier formation at endosomes was only stimulated in the presence of cytosol. Wild-type Rab11 had no significant effect on carrier formation while the dominant negative (S22N) and constitutively active (Q70L) slightly decreased carrier formation of both TfR and EGFR containing carriers. Since both Rab11 S22N and Q70L have an inhibiting effect on carrier formation, it is likely

that not only active Rab11 but also GTP hydrolysis is required. This is consistent with *in vivo* experiments that showed both of these mutants to reduce the recycling rate of the TfR (Ren et al., 1998; Ullrich et al., 1996).

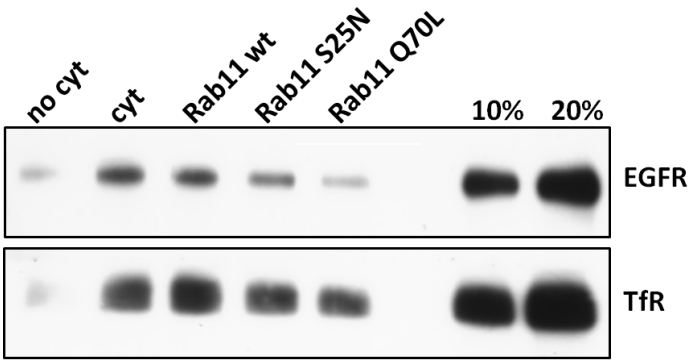

Figure 16. Rab11 is involved in carrier formation at endosomes.

Biotinylated permeabilized BHK cells were incubated in the presence of ATP, GTP, and ATP-regenerating system without cytosol (no cyt), with cytosol (cyt), with cytosol containing wild type Rab11 (Rab11 wt), with cytosol containing dominant negative Rab11 (Rab11 S25N) or with cytosol containing constitutively active Rab11 (Rab11 Q70L). Immunoblot analysis of biotinylated EGFR and TfR in the supernatant after cell pelleting is shown. 10% and 20% of the total biotinylated material are shown as a control.

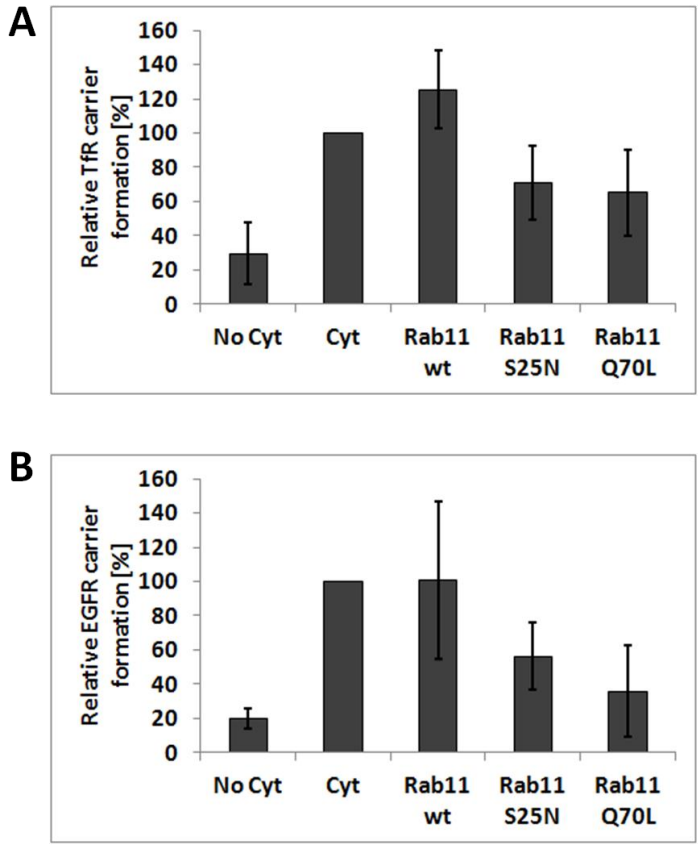

Figure 17. Quantitation of the effect of Rab11 on endosomal TfR and EGFR containing carrier formation.

(A) Rab11 wild-type has no significant effect on the formation of carriers containing TfR (N = 4) while dominant negative (N = 6) and constitutively active (N = 5) Rab11 significantly inhibit carrier formation.
(B) Rab11 wild-type has no significant effect on the formation of carriers containing EGFR (N = 3) while dominant negative (N = 4) and constitutively active (N = 2) Rab11 significantly inhibit carrier formation.

Rab14 is Not Involved in the Formation of Transport Carriers at Endosomes

Rab14 localizes to the TGN and endosomes and was found together with Rab4 and partially with Rab11 in gradient fractionationing experiments (Junutula et al., 2004; Proikas-Cezanne et al., 2006). Whether Rab14 is involved in recycling is not entirely clear, since one study showed Rab14 to have no effect on TfR recycling, whereas another study did (Junutula et al., 2004; Yamamoto et al., 2010). Furthermore, Rab14 has been shown to be able to interact with the Rab4 effector Rabip4 and the Rab11 effector family of class 1 FIPs (Kelly et al., 2010; Yamamoto et al., 2010). We were therefore interested to test whether Rab14 plays a role in carrier formation at endosomes. To test this we performed an endosomal transport carrier formation assay with cytosol from HeLa T-REx cells expressing wild-type or dominant negative Rab14 and from untransfected HeLa T-REx cells. The assay was carried out as described above. As shown in Figure 18 carrier formation at endosomes was

equally stimulated by the presence of normal cytosol, cytosol containing overexpressed wild-type (wt) Rab14, and cytosol containing dominant negative (S25N) Rab14. Further experiments in our lab confirm these findings, and also show constitutive active Rab14 not to affect carrier formation in comparison to cytosol from untransfected cells (Barry Shortt, unpublished data).

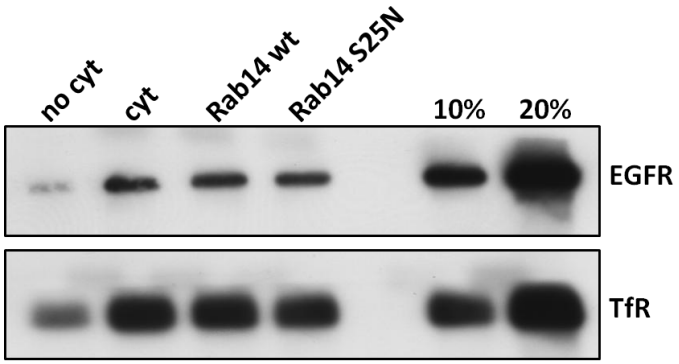

Figure 18. Rab14 is not involved in carrier formation at endosomes.

Biotinylated permeabilized BHK cells were incubated in the presence of ATP, GTP, and ATP-regenerating system without cytosol (no cyt), with normal cytosol (cyt), with cytosol containing wild type Rab14 (Rab14 wt) or with cytosol containing dominant negative Rab14 (Rab14 S25N). Immunoblot analysis of biotinylated EGFR and TfR in the supernatant after cell pelleting is shown. 10% and 20% of the total biotinylated material are shown as a control.

Part II: *In Vivo* Recycling of the Transferrin Receptor

In vitro techniques like the carrier formation assay described above are great tools to dissect the secretory system and examine specific transport steps. However, it is advantageous to combine *in vitro* with *in vivo* experiments, and to complement data. To have a tool ready in the lab and to further analyze the *in vivo* role of Rab4 and Rab11, especially since the role of Rab4 is not entirely clear, a novel recycling and endocytosis assay was established.

Establishing a Novel Recycling and Endocytosis Assay

To investigate whether a protein is involved in endocytosis, one often measures the uptake rate of a ligand or receptor. In an endocytosis assay, cells are often provided with a labeled ligand and the endocytosis rate can be determined by measuring the intracellular appearance of the ligand over time. To investigate whether a protein is involved in recycling from endosomes to the plasma membrane, it is usually necessary to follow a receptor, since most ligands do not recycle, but are instead degraded. In a recycling assay, receptors at the plasma membrane have to be labeled first and are then allowed to be endocytosed for a specific amount of time. The recycling rate can then be determined by measuring disappearance of the labeled receptor from the cells over time. To measure the recycling rate accurately, re-endocytosis of labeled receptors needs to be prevented. The TfR and its ligand Tf have become a preferred tool to study constitutive endocytosis and recycling, because Tf is recycled together with its receptor, eliminating the issue of labeling, and later preventing re-endocytosis of a receptor. Tf is a secretory protein that binds two Fe^{3+} ions with high-affinity. Iron loaded holo-Tf is bound by the TfR and rapidly endocytosed. In the more acidic environment of early sorting endosomes, the iron is released from Tf. However, unlike most other ligands (for example LDL) apo-Tf remains bound to its receptor. The apo-Tf-TfR complex is then recycled back to the plasma membrane either directly from early sorting endosomes or via recycling endosomes. At the neutral extracellular pH, iron-free apo-transferrin is released from the receptor (Ciechanover et al., 1983; Maxfield and McGraw, 2004; Stein and Sussman, 1986). In steady-state, approximately 80% of the TfR is intracellular, while 20% is at the plasma membrane (Davis et al., 1986; Klausner et al., 1983; van der Sluijs et al., 1992).

To be able to follow trafficking of a specific ligand, this ligand needs to be labeled. The first method established, was to label Tf with radioactive iodine. The advantage of this method is the high sensitivity of the assay and the fact that one can easily measure the amount of ligand inside the cell, at the plasma membrane, and in the chase medium. Another possibility is to use biotinylated Tf. In this case only internal Tf can be easily measured, for example by detecting the biotinylated Tf with

streptavidin-HRP either after SDS gel electrophoresis and blotting, or directly on the culture dish with a microplate reader. The third possibility is to use fluorescent transferrin and measure the fluorescence intensity of single cells. This can be done either with flow cytometry or fluorescence microscopy.

We decided to use fluorescently labeled Tf and fluorescence microscopy for our endocytosis and recycling assays. To improve the assay, we made use of automated immunofluorescence imaging and image analysis software. By using this automated analysis process, it is possible to measure the fluorescence intensity of a labeled ligand, in our case Tf, in thousands of individual cells at different time points. Individual cells were identified by staining the nuclei with the DNA dye Hoechst and the actin cytoskeleton with fluorescently labeled phalloidin. By combining nuclei (primary objects) and the actin cytoskeleton (secondary objects) the image analysis software Cell Profiler is able to identify single cells. An example of how individual cells were detected can be found in Figure 19.

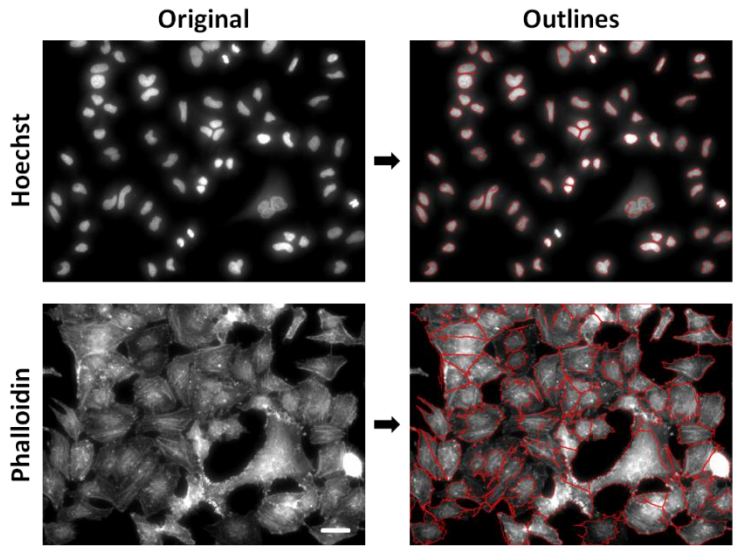

Figure 19. Automated detection of individual cells by Cell Profiler.

HeLa CCL-2 cells were fixed with 3% PFA, permeabilized with 0.1% Triton-X-100, the nuclei were stained with Hoechst and the actin cytoskeleton with fluorescently labeled phalloidin. Hoechst staining was used to identify primary objects (Nuclei) and phalloidin staining to identify the secondary objects around the nuclei (cells). Scale bar represents 20 µm.

For an endocytosis assay, cells were starved for 2 h in serum-free medium to deplete endogenous Tf. They were incubated in the presence of fluorescently labeled transferrin for various times. To stop endocytosis the cells were rapidly cooled to 4°C and washed with ice cold PBS and acidic wash buffer to remove surface bound transferrin. The cells were then fixed with paraformaldehyde and permeabilized with Triton-X-100. The nucleus was stained with Hoechst and the actin cytoskeleton with fluorescently labeled phalloidin.

For a recycling assay, cells were starved for 2 h in serum-free medium to deplete endogenous Tf. They were then incubated in the presence of fluorescently labeled transferrin for 1 h to load the TfR to steady-state. The cells were then cooled to 4°C, washed with ice cold PBS and acidic wash buffer to remove surface bound transferrin. After that the cells were warmed to 37°C in the presence of chase medium containing 50 µM of the iron chelating agent deferoxamine for various times, to prevent rebinding of Tf to the TfR. To stop the recycling reaction, the cells were fixed with paraformaldehyde, then cooled to 4°C and washed with ice cold PBS. The cells were then permeabilized with Triton-X-100, the nucleus was stained with Hoechst and the actin cytoskeleton with fluorescently labeled phalloidin.

Evaluation of Different Acidic Wash Buffers

The first issue was to find a suitable acidic wash buffer to remove surface bound transferrin. We tested two different stripping procedures described in the literature. The first stripping solution was a MES buffer at pH 5.5 (150 mM NaCl, 50 mM MES (2-(N-morpholino)ethanesulfonic acid), pH 5.5), the second a glycine buffer at pH 2 (150 mM glycine, pH 2) (Gong et al., 2007; Inoue et al., 2008). To test the efficiency of these acidic wash buffers, HeLa cells were incubated with 10 µg/ml biotinylated Tf for 1 h at 4°C. The cells were washed with ice cold PBS and an acidic wash buffer, or only with PBS. After that they were lysed, and the presence of biotinylated Tf was detected with streptavidin-HRP after SDS gel electrophoresis and blotting. As shown in Figure 20A, the removal of Tf from the cell surface was very efficient in the case of the glycine buffer at pH 2, while the MES buffer at pH 5.5 was ineffective. However, it soon became apparent that cells treated with the glycine buffer at pH 2 were not stained by phalloidin anymore (Figure 20B). To look at the state of the actin cytoskeleton in glycine buffer treated cells, HeLa cells were fixed with paraformyldehyde, permeabilized with Triton-X-100 and stained with the anti-actin antibody 2g2 IgM and FITC-conjugated goat anti-mouse IgM as a secondary antibody. As shown in Figure 20B, the actin staining pattern of untreated cells looked similar with phalloidin and the antibody. However, in cells treated with the glycine buffer at pH 2, phalloidin staining was absent, and the staining of the actin antibody clearly showed an alteration of the distribution and the state of actin in the cells. Since we could not use this acidic wash buffer, we chose to test a sodium acetate buffer at pH 3.5 (150 mM NaCl, 50 mM NaOAc, pH 3.5) (Verdier et al., 2000). A glycine buffer at pH 4.5 (150 mM glycine, pH 4.5) and saline at pH 3.35 (154 mM NaCl, pH 3.35) were also tested. We determined the efficiency of these acidic wash buffers as described above. As shown in Figure 20C, the sodium acetate buffer at pH 3.5 had the same efficiency in removing surface bound Tf as the glycine buffer at pH 2, but did not alter the actin cytoskeleton (Figure 20D). Saline at pH 3.35 and the glycine buffer at pH 4.5 were slightly less efficient in removing

surface bound Tf. Therefore we continued to use the sodium acetate buffer at pH 3.5 as acidic wash buffer in the subsequent experiments.

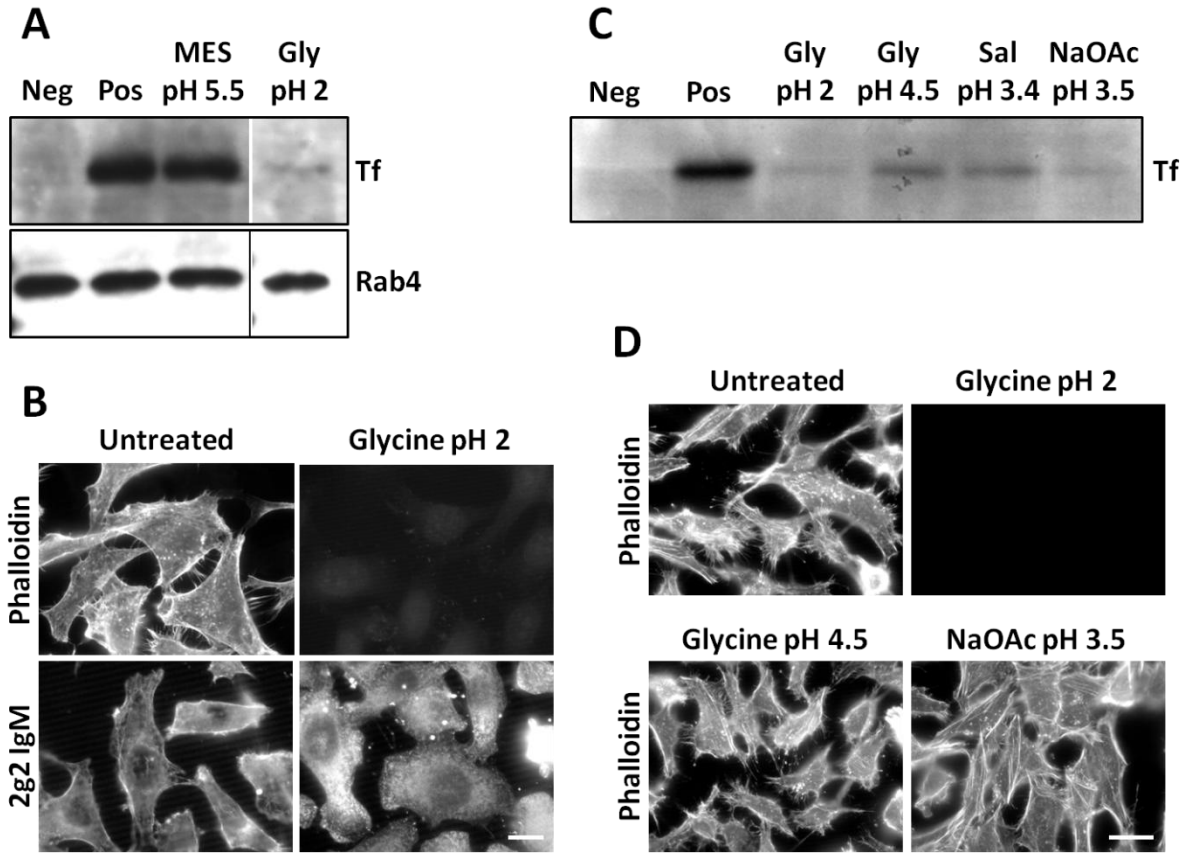

Figure 20. Evaluation of different acidic wash buffers

(A) HeLa cells were either incubated with PBS (Neg) or with PBS containing Biotin-Tf (Pos) at 4°C. Cells that had been incubated with Biotin-Tf were then washed with either 150 mM NaCl, 50 mM MES, pH 5.5 (MES pH 5.5) or 150 mM glycine, pH 2 (Gly pH 2). Cells were then lysed and the presence of Biotin-Tf was assessed by SDS gel electrophoresis and streptavidin blotting. Lanes separated by white or black lines are from the same blot.
(B) HeLa cells were either washed with PBS (Untreated) or with 150 mM glycine, pH2 (Glycine pH 2). The cells were then fixed with 3% PFA and either stained with fluorescent phalloidin or the anti-actin antibody 2g2. Scale bar represents 10 µm.
(C) HeLa cells were either incubated with PBS (Neg) or with PBS containing Biotin-Tf (Pos) at 4°C. Cells that had been incubated with Biotin-Tf were then washed with either 150 mM glycine, pH 2 (Gly pH2), 150 mM glycine, pH 4.5 (Gly pH 4.5), 154 mM NaCl (Sal), or 150 mM NaCl, 50 mM NaOAc, pH 3.5 (NaOAc pH 3.5). Cells were then lysed and the presence of Biotin-Tf was assessed by SDS gel electrophoresis and streptavidin blotting.
(D) HeLa cells were either washed with PBS (untreated), 150 mM glycine, pH 2 (Glycine pH 2), 150 mM glycine, pH 4.5 (Glycine pH 4.5) or 150 mM NaCl, 50 mM NaOAc, pH 3.5 (NaOAc). The cells were then fixed with 3% PFA and either stained with fluorescent phalloidin. Scale bar represents 20 µm.

The Recycling and Endocytosis Assay

As a proof of concept we performed our recycling and endocytosis assay as described above with HeLa CCL-2 cells. To determine the fluorescence intensity per cell, individual cells were automatically identified by Cell Profiler, and the mean fluorescence intensity per cell was measured. The mean intensity is the average intensity of all the pixels in one cell. Alternatively it is possible to measure the integrated intensity. The integrated intensity is the sum of all pixel intensities per cell. Whether the mean or the integrated intensity was measured is indicated for every individual graph. As shown in Figure 21A transferrin was endocytosed with a half time of approximately 3 to 4 min, assuming that steady state distribution of fluorescent transferrin was reached after 20 min. In the recycling experiment about half the transferrin was chased from the cells after approximately 7.5 min (Figure 21C). These numbers are similar to previously determined recycling kinetics of Tf (Ciechanover et al., 1983). Furthermore, we wanted to test whether we could observe defects in endocytosis and recycling by using Dynasore and the ionophore Monensin. Dynasore is a potent inhibitor of dynamin dependent endocytosis and is able to almost completely block Tf uptake (Macia et al., 2006). Monensin is able to almost completely block Tf recycling from endosomes to the plasma membrane (Stein et al., 1984). As shown in Figure 21B+D we could observe an almost total block of Tf endocytosis in the presence of Dynasore and a strongly reduced recycling rate of Tf in the presence of Monensin.

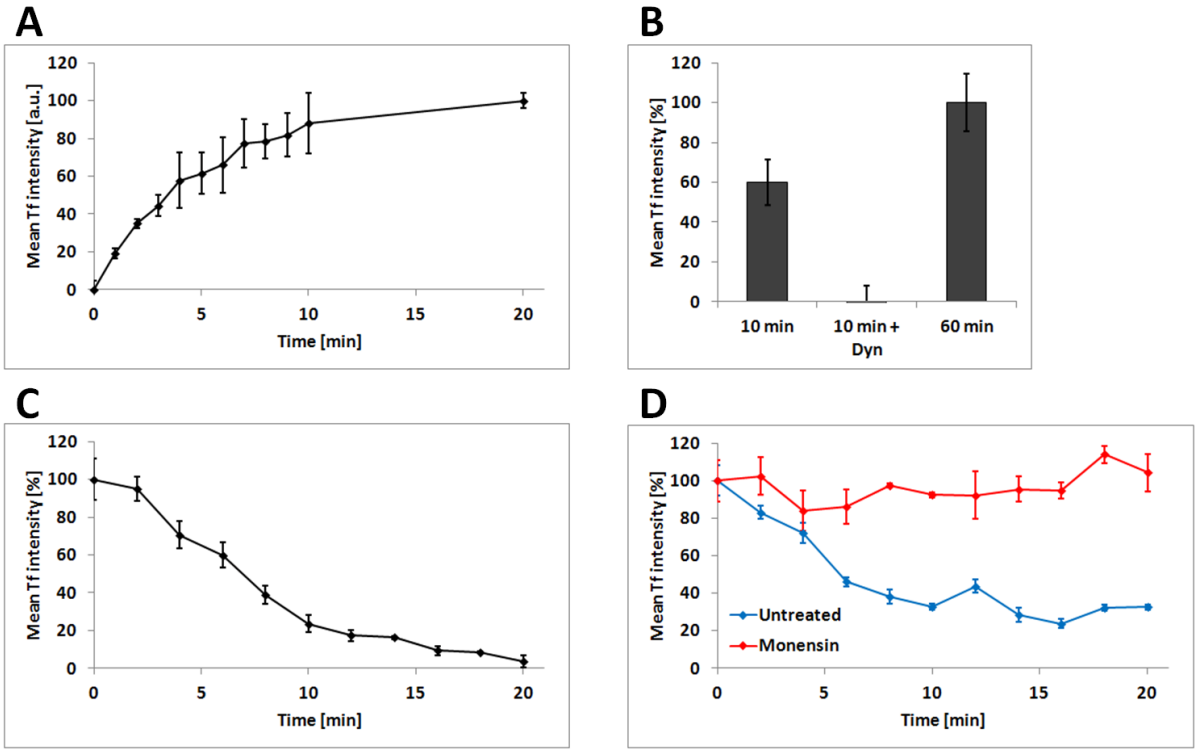

Figure 21. The *in vivo* recycling and endocytosis assays

(A) Endocytosis assay. HeLa cells were loaded with fluorescent transferrin, endocytosis was stopped every min for 10 min and after 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and the standard deviation of 3 to 4 assay wells. Similar results have been observed in 3 independent experiments.
(B) HeLa cells were either loaded for 10 min or 60 min with fluorescent Tf or for 10 min with fluorescent Tf in the presence of 80 µM dynasore. Mean Tf intensity per cell was measured. Graph represents the mean value and the standard deviation of every cell per condition of one individual experiment.
C) Recycling assay. HeLa cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells, recycling was stopped after every 2 min up to 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and the standard deviation of 4 assay wells. Similar results have been observed in 3 independent experiments.
(D) HeLa cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells either not in the presence or in the presence of 10 µM monensin, recycling was stopped after every 2 min up to 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and the standard deviation of 4 assay wells. Similar results have been observed in 3 independent experiments.

It should be noted, that there is an obvious variability in the recycling rate between individual experiments. In Figure 22 three independent recycling experiments (Figure 22A, B and C) and the mean values of the three experimets (Figure 22D) are depicted. There is an obvious difference of over 10% between some data points of the 3 individual experiments. It is therefore necessary to only directly compare cells on the same assay plate and not between assay plates. This means that uninduced and induced or untransfected and transfected cells can only be compared when they are on the same plate, being treated in the same way and at the same time.

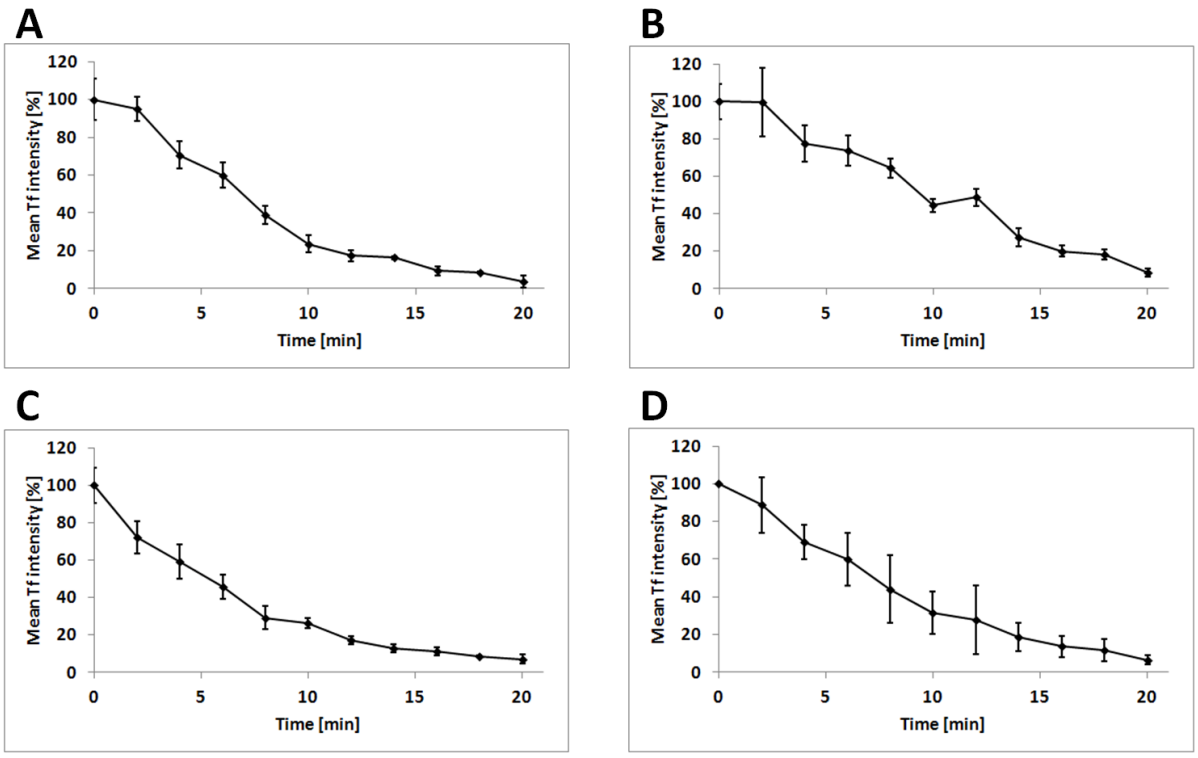

Figure 22. Variations of the recycling rate in three independent experiments performed in HeLa cells.

(A) to (C) HeLa cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells, recycling was stopped after every 2 min up to 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and the standard deviation of 4 assay wells
(D) Mean value and standard deviation of the 3 individual experiments shown in (A) to (C).

HeLa T-REx Cells are Sensitive to Stress

Next we wanted to use our HeLa T-REx cells expressing the various Rabs and mutants in the endocytosis and recycling assay. Surprisingly, as shown in Figure 23, an increase of mean intracellular Tf fluorescence intensity was observed in the first few minutes of recycling, instead of the expected decrease. We discovered that cells subjected to chase medium no longer had typical fibroblast morphology, but were smaller and round (Figure 23C+D). This explained the increase of mean Tf fluorescence intensity, since we measured the average pixel intensity within an object (mean

intensity of a cell). This means that cells that have taken up the same amount of fluorescent transferrin will appear to have higher mean fluorescence intensity when they become smaller, because the transferrin will be packed more densely. Indeed, when measuring the sum of the pixel intensities within an object (integrated intensity of a cell), an increase of intracellular Tf fluorescence intensity in the first few minutes of recycling was not observed, because the rounded cells have brighter, but less, pixels (Figure 23B). However, the integrated intensity of fluorescent transferrin dropped below zero in the example depicted here, because the cells used to determine background fluorescence were normal size, therefore having more pixels with background fluorescence that were measured.

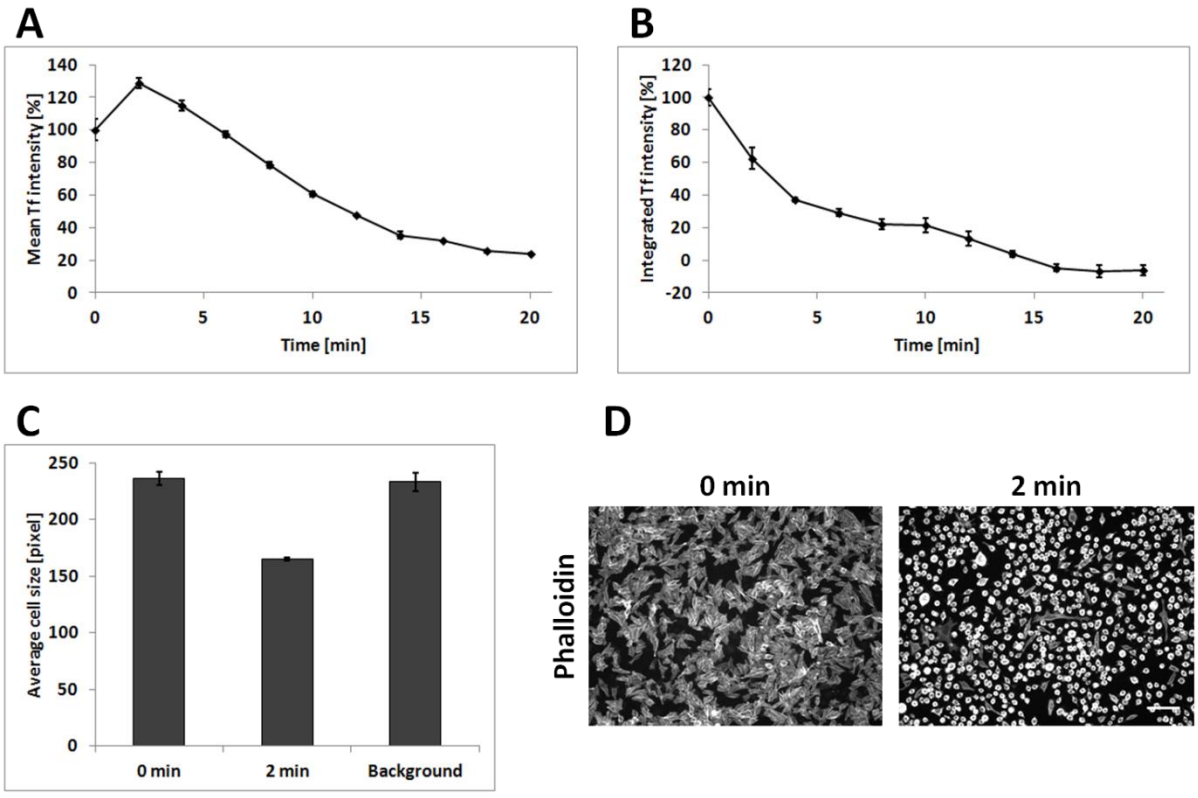

Figure 23. HeLa T-REx cells round up, causing an increase in mean Tf intensity during the recycling procedure.

(A) HeLa T-REx cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells, recycling was stopped after every 2 min up to 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and the standard deviation of 4 assay wells.
(B) The exact same experiment as (A). Integrated intensity instead of mean intensity per cell was measured. Graph represents the mean value and the standard deviation of 4 assay wells.
(C) Average cell size in pixels of the cells at time point 0 min, time point 2 min, and the cells used to determine background fluorescence in the same experiment as (A). Graph represents the mean value and the standard deviation of 4 assay wells. Cells at time point 2 min are significantly smaller than at time point 0 min, p = 3.5E-7.
(D) Phalloidin staining of the cells at time point 0 min and at time point 2 min in the same experiment as (A). Scale bar represents 50 µm.

Since only cells subjected to chase medium were becoming smaller and round, deferoxamine was first suspected to be harmful to HeLa T-REx cells. To investigate this, HeLa T-REx cells were incubated for 1 h at 37°C in medium containing 20 mM HEPES and with or without 50 µM deferoxamine. The cells were then imaged and the cell size in number of pixels was measured. As shown in Figure 24A deferoxamine had no negative effect on cell morphology. Next we tested whether fixing cells at 37°C caused the cells to become smaller and round. Therefore we fixed HeLa T-REx cells at 4°C and at 37°C and then imaged the cells and again measured cell size. As shown in Figure 24B there was a slight decrease in cell size if cell size were fixed at 37°C in comparison to cells fixed at 4°C. Finally we tested the influence of cooling cells to 4°C and warming them to 37°C. We could observe that the HeLa T-REx cells became smaller and round when they were warmed from 4°C to 37°C (Figure 24C). However, the same was not true for HeLa CCL-2 cells (Figure 24D).

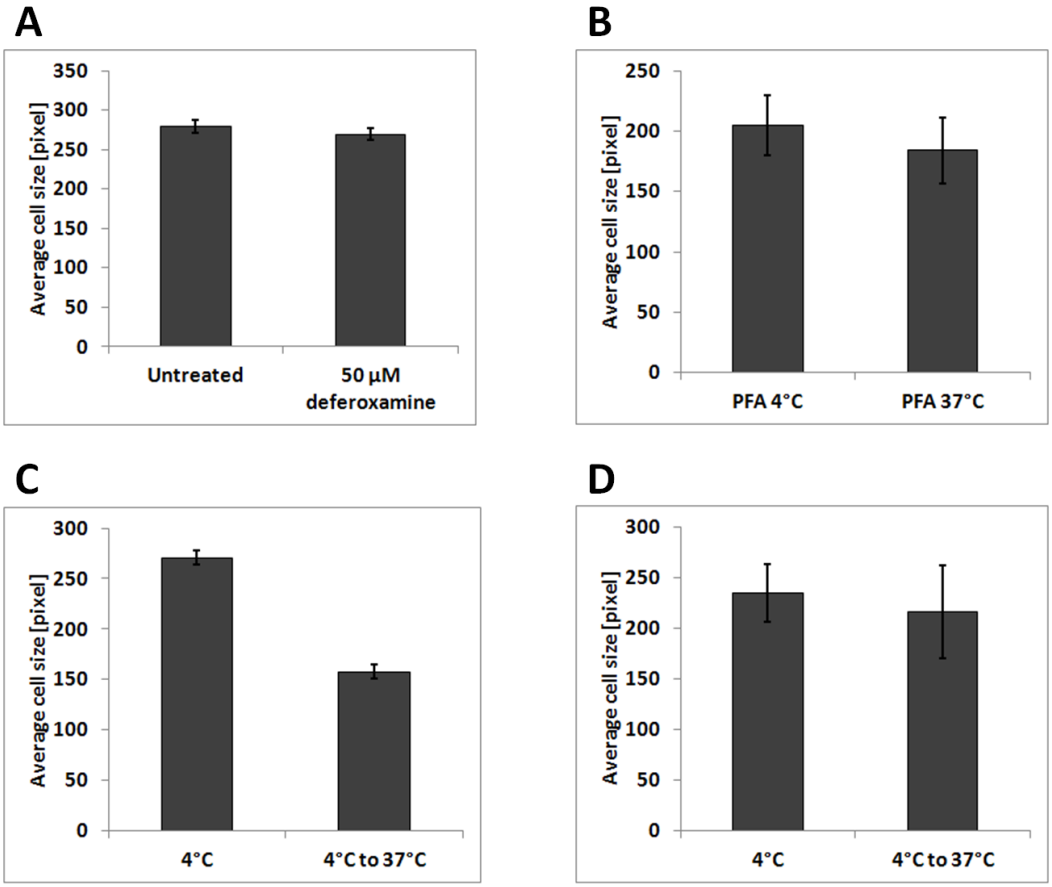

Figure 24. The cause of the HeLa T-REx cells rounding up.

(A) HeLa T-REx cells were either incubated in normal medium or in medium containing 50 µM deferoxamine for 1 h. Average cell size in pixels was measured. Graph represents the mean value and the standard deviation of 16 wells of an individual experiment.
(B) HeLa T-REx cells were either fixed with 3% paraformaldehyde (PFA) at 4°C or at 37°C. Average cell size in pixels was measured. Graph represents the mean value and the standard deviation of 8 wells per condition of an individual experiment, p = 0.067.
(C) HeLa T-REx cells were either fixed with 3% PFA at 4°C or cooled to 4°C with ice cold PBS, warmed to 37°C and then fixed with 3% PFA. Average cell size in pixels was measured. Graph represents the mean value and standard deviation of 8 assay wells, p = 1.6E-14.
(D) HeLa CCL-2 cells were either fixed with 3% PFA at 4°C or cooled to 4°C with ice cold PBS, then warm to 37°C and then fixed with 3% PFA. Average cell size in pixels was measured. Graph represents the mean value and standard deviation of 8 assay wells, p = 0.34.

Since it was not possible to conduct experiments with HeLa T-REx cells that required a step in which they are warmed from 4°C to 37°C, the protocol for the recycling assay was modified. The cells were starved for 2 h in serum-free medium to deplete endogenous Tf, and then incubated in the presence of fluorescently labeled Tf for 1 h. After that the cells were incubated in the presence of chase medium containing 50 µM deferoxamine for various times. To stop the recycling reaction, the cells were quickly cooled to 4°C with ice cold PBS and then fixed with paraformaldehyde. The cells were then permeabilized with Triton-X-100, stained with Hoechst and fluorescently labeled phalloidin.

Overall Transferrin Recycling is Not Affected by Manipulating Rab4 Activity

Rab4 is generally assumed to be a major regulator of the fast recycling pathway from early sorting endosomes to the plasma membrane. However the literature is not entirely clear on the role of Rab4. Rab4 has been reported to not affect release of previously internalized Tf from CHO cells (van der Sluijs et al., 1992). In contrast to these findings, it was reported that overexpression of dominant negative (S22N) Rab4 led to a 30% inhibition of Tf recycling, while wild-type and constitutively active (Q67L) Rab4 led to a 10% increase of Tf recycling in HeLa cells (data not shown in McCaffrey et al., 2001). Additionally, there are two conflicting reports about the effect of siRNA mediated knockdown of Rab4 in HeLa cells. In one report Tf was shown to recycle faster in knockdown cells, while the other report showed Tf recycling to be impaired (Deneka et al., 2003; Yamamoto et al., 2010).

Since the role of Rab4 in TfR recycling is not clear, we used our HeLa T-REx cell lines expressing dominant negative (S22N, N121I) and constitutively active (Q67L) Rab4 to perform recycling assays. Cells were allowed to take up fluorescent Tf for 1 h to fill the endosomal compartments to steady state. The Tf was then chased from the cells in the presence of 50 µM deferoxamine, and recycling was stopped every 2 min for 20 min. As shown in Figure 25, Rab4 S22N did not affect the recycling rate of Tf. Four independent experiments are shown in Figure 25 to illustrate the inter-assay variability described before again. Rab4 Q67L (Figure 26A) and N121I (Figure 26B) also had no inhibiting or stimulating effect on Tf recycling. In the case of the Rab4 Q67L and NI121I experiments (Figure 26), the integrated intensity is shown instead of the mean intensity, because all of the individual experiments were subject to the stress induced shrinking of the HeLa T-REx cells and therefore the mean intensity appeared to increase in the first few minutes of recycling.

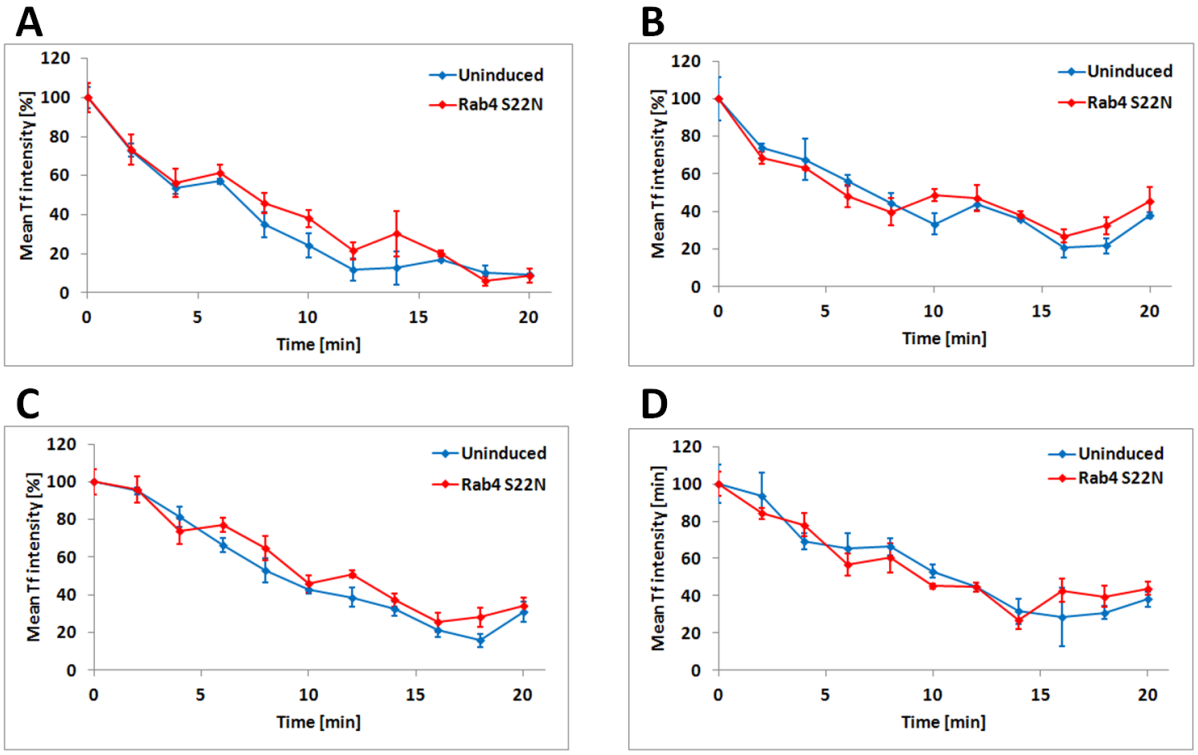

Figure 25. Dominant negative S22N Rab4 in transferrin recycling: Four independent experiments.

(A) to (D) HeLa T-REx cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells and recycling was stopped after every 2 min up until 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and standard deviation of 3 to 4 assay wells. 4 independent experiments are shown.

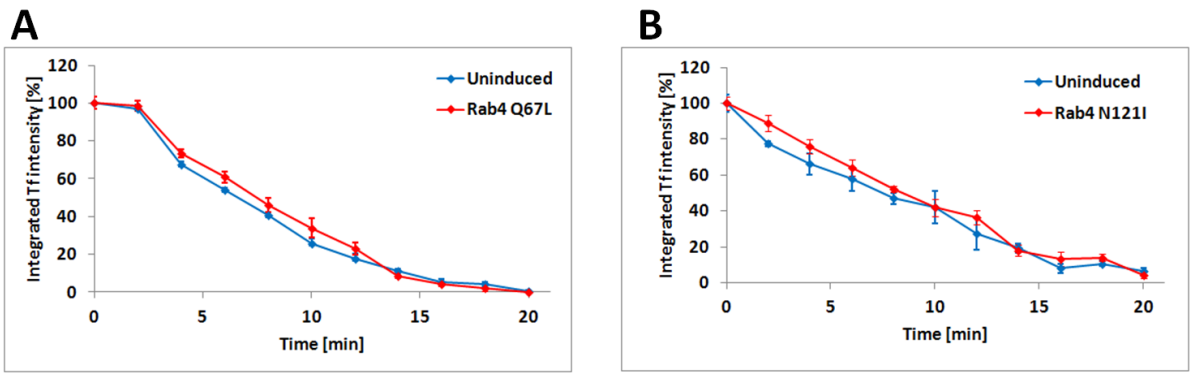

Figure 26. Constitutively active Q67L and dominant negative N121I Rab4 in transferrin recycling.

(A) HeLa T-REx cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells and recycling was stopped after every 2 min up to 20 min. Integrated Tf intensity per cell was measured. Graph represents the mean value and standard deviation of 3 to 4 assay wells. Similar results have been observed in 2 independent experiments.
(B) HeLa T-REx cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells and recycling was stopped after every 2 min up to 20 min. Integrated Tf intensity per cell was measured. Graph represents the mean value and standard deviation of 4 assay wells. Similar results have been observed in 4 independent experiments

Furthermore, we wanted to investigate the effect of Rab4 knockdown on Tf recycling. Therefore we transfected HeLa cells with siRNA directed against Rab4. Knockdown was approximately 90% as determined by semi-quantitative immunoblot analysis (Figure 27B). A recycling assay was performed 72 h post transfection. As shown in Figure 27A knockdown of Rab4 had no effect on the kinetics of Tf recycling from preloaded cells.

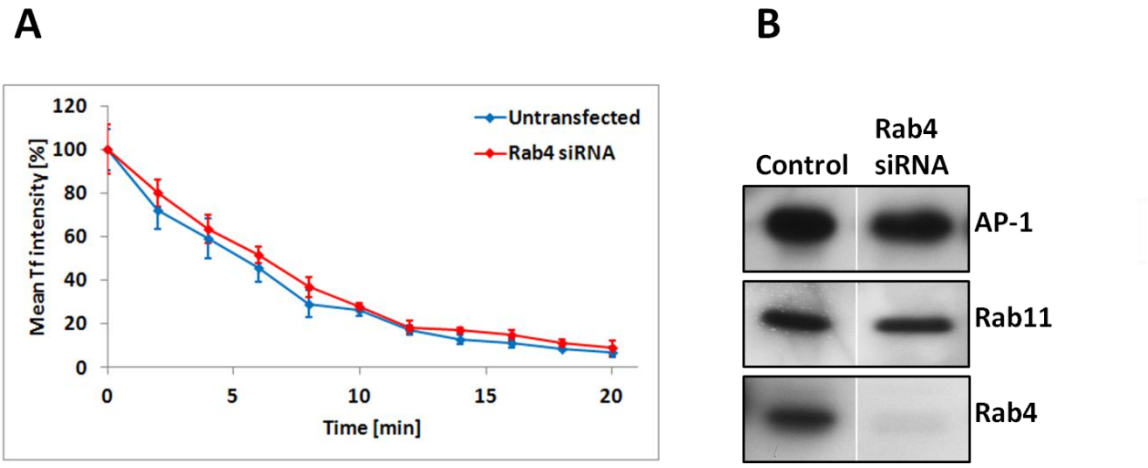

Figure 27. Rab4 knockdown.

(A) HeLa cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells and recycling was stopped after every 2 min up to 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and standard deviation of 4 assay wells. Similar results have been observed in 3 independent experiments.
(B) HeLa cells were either transfected with siRNA directed against Rab4 (Rab4 siRNA) or not transfected (control). Total cell lysates were produced 72 h post transfection and the level of knock-down was assessed by SDS gel electrophoresis and immunoblotting. AP-1 and Rab11 were used as a loading control. Lanes separated by white lines are from the same blot.

Overall Transferrin Recycling is Not Affected by Manipulating Rab11 Activity

Rab11 localizes to perinuclear recycling endosomes where it has been implicated to be involved in the regulation of the slow recycling pathway from these endosomes to the plasma membrane. Tf recycling was reported to be moderately slowed in cells expressing dominant negative Rab11 (S25N) and minimally reduced in cells expressing constitutively active (Q70L) or overexpressing wild-type Rab11 (Ren et al., 1998; Ullrich et al., 1996).

To investigate whether Rab11 affected Tf recycling, we used our HeLa T-REx cells expressing wild-type, S25N or Q70L Rab11, or shRNA directed against Rab11, to perform recycling assays. The cells were allowed to take up fluorescent transferrin for 1 h, after that Tf was chased in the presence of 50 µM deferoxamine, and the recycling reaction was stopped at indicated time points. As shown in Figure 28 neither overexpression of wild-type or mutant Rab11 nor Rab11 knockdown significantly altered the rate of Tf recycling compared to uninduced cells.

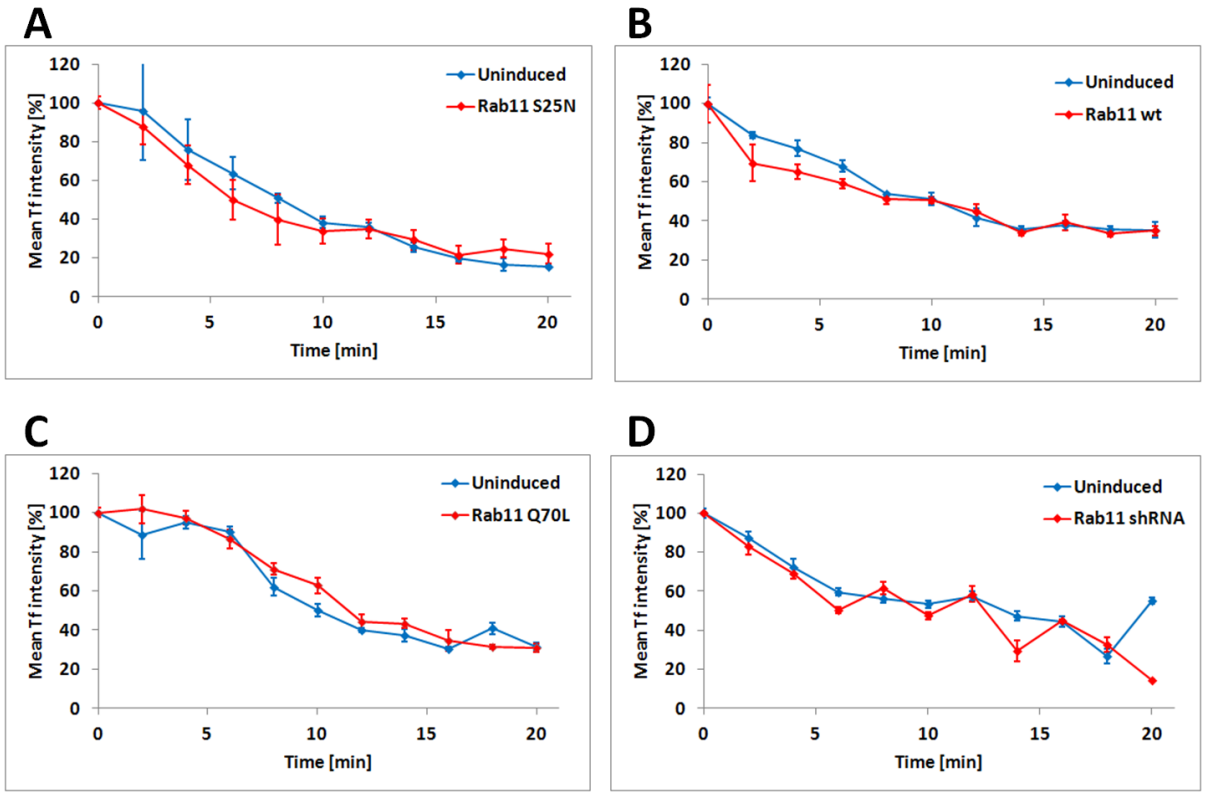

Figure 28. Rab11 in transferrin recycling.

(A) HeLa T-REx cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells and recycling was stopped after every 2 min up to 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and standard deviation of 3 to 4 assay wells. Similar results have been observed in 3 independent experiments
(B) HeLa T-REx cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells and recycling was stopped after every 2 min up to 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and standard deviation of 3 to 4 assay wells.
(C) HeLa T-REx cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells and recycling was stopped after every 2 min up to 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and standard deviation of 2 to 4 assay wells.
(D) HeLa T-REx cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells and recycling was stopped after every 2 min up to 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and standard deviation of 3 to 4 assay wells. Similar results have been observed in 3 independent experiments.

Since neither Rab4 nor Rab11 had a substantial effect on recycling of Tf, we wanted to investigate whether this was only the case in HeLa T-REx or also in other HeLa cells. Therefore we transfected HeLa cells with dominant negative Rab11 (S25N) coupled with GFP. Rab11 S25N was chosen because it was shown to have the strongest negative effect on recycling in BHK and CHO cells (Ren et al., 1998; Ullrich et al., 1996). By using GFP coupled to Rab11 S25N, it was possible to detect transfected cells during image analysis by measuring green fluorescence. Cells exhibiting green fluorescence above a certain threshold were used to measure Tf intensity of transfected cells while cells exhibiting green fluorescence below a certain threshold were used to measure Tf intensity of untransfected cells. Between the two thresholds there was a gap to clearly distinguish between transfected and untransfected cells. In Figure 29A transfected cells are shown. It should be noted, that the

transfection efficiency was poor, with only just over 100 transfected cells per time point in the worst case. The recycling assay was performed 24 h post transfection. As shown in Figure 29B expression of Rab11 S25N in HeLa cells did not alter the Tf recycling rate, indicating that our results obtained in HeLa T-REx cells are also valid for HeLa cells. Two independent experiments are shown.

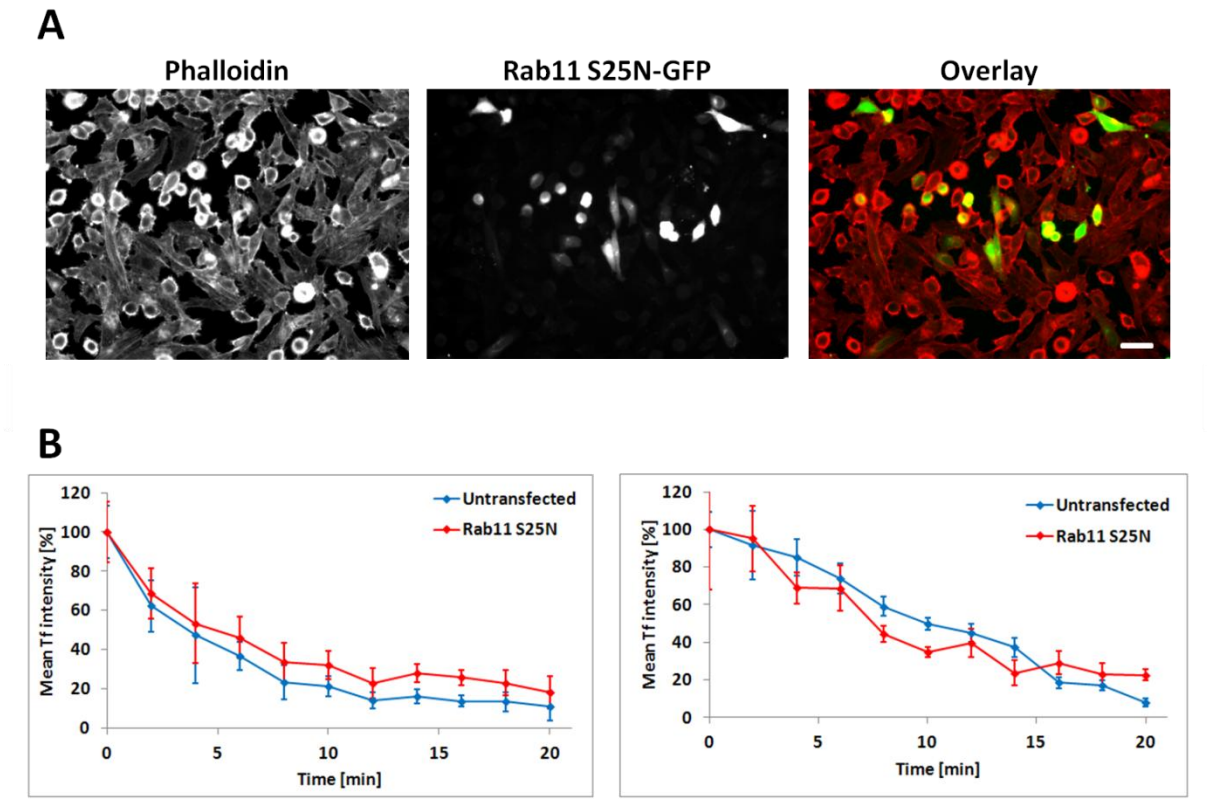

Figure 29. Dominant negative Rab11 has no influence on Tf recycling in HeLa CCL-2 cells.

(A) Representative image of cells transfected with Rab11 S25N-GFP (green). The actin cytoskeleton was stained with Alexa-647-phalloidin (red). Scale bar represent 20 µm.
(B) HeLa cells were loaded with fluorescent Tf for 1 h. Tf was then chased from the cells and recycling was stopped after every 2 min up to 20 min. Mean Tf intensity per cell was measured. Graph represents the mean value and standard deviation of 6 to 8 assay wells.

Overexpression of Rab4 and Rab11 do Not Influence the Expression Levels of Each Other

Since neither mutant expression, nor gene silencing of Rab4 and Rab11 significantly affected overall Tf recycling in our assays, we wanted to investigate whether impairment of one Rab dependent pathway would lead to the up-regulation of other Rab dependent pathways. As mentioned above, Tf is recycled directly in the fast recycling pathway from sorting endosomes to the plasma membrane or in the slow recycling pathway via recycling endosomes. It is possible that Tf is trafficked to a greater extent through the slow recycling pathway when the fast recycling pathway is impaired and vice versa. To compensate for the increased cargo load in one pathway, an increased activity of Rab proteins might be necessary. This could be achieved through increased activity of the Rab protein, or

by increased gene expression. To test this, expression levels of Rab4, Rab11, and Rab7 in the HeLa T-REx cells overexpressing wild-type, dominant negative and constitutively active Rab4 and Rab11 were checked. Rab7 localizes to late endosomes and mediates late endosome maturation and fusion with lysosomes, and was used as a negative control (Bucci et al., 2000; Feng et al., 2001).

HeLa T-REx cells expressing wild-type, dominant negative and constitutively active Rab4 and Rab11 were either induced or not induced for 24 h with doxycycline. The cells were then lysed and equal amounts of total protein were analyzed for Rab4, Rab11, and Rab7 expression levels by SDS gel electrophoresis and immunoblotting. As shown in Figure 30A, overexpression of Rab4 wild-type, S22N, N121I, or Q67L did not significantly affect expression levels of Rab11 and Rab7 in an obvious manner. Similarly overexpression of Rab11 wild-type, S25N, and Q70L did also not affect expression levels of Rab4 and Rab7 (Figure 30B). These results indicate that impairment of a one Rab does not lead to compensatory increased expression levels of another Rab protein. However, it cannot be excluded that other factors involved in recycling are up-regulated to compensate impaired Rab4 or Rab11 function. For example, the two Rab proteins Rab15 and Rab35 have been implicated to be somehow involved in recycling (Kouranti et al., 2006; Zuk and Elferink, 2000). Furthermore, different pathways could have sufficient capability to compensate impaired Rab4 or Rab11 function without up-regulated gene expression of Rabs.

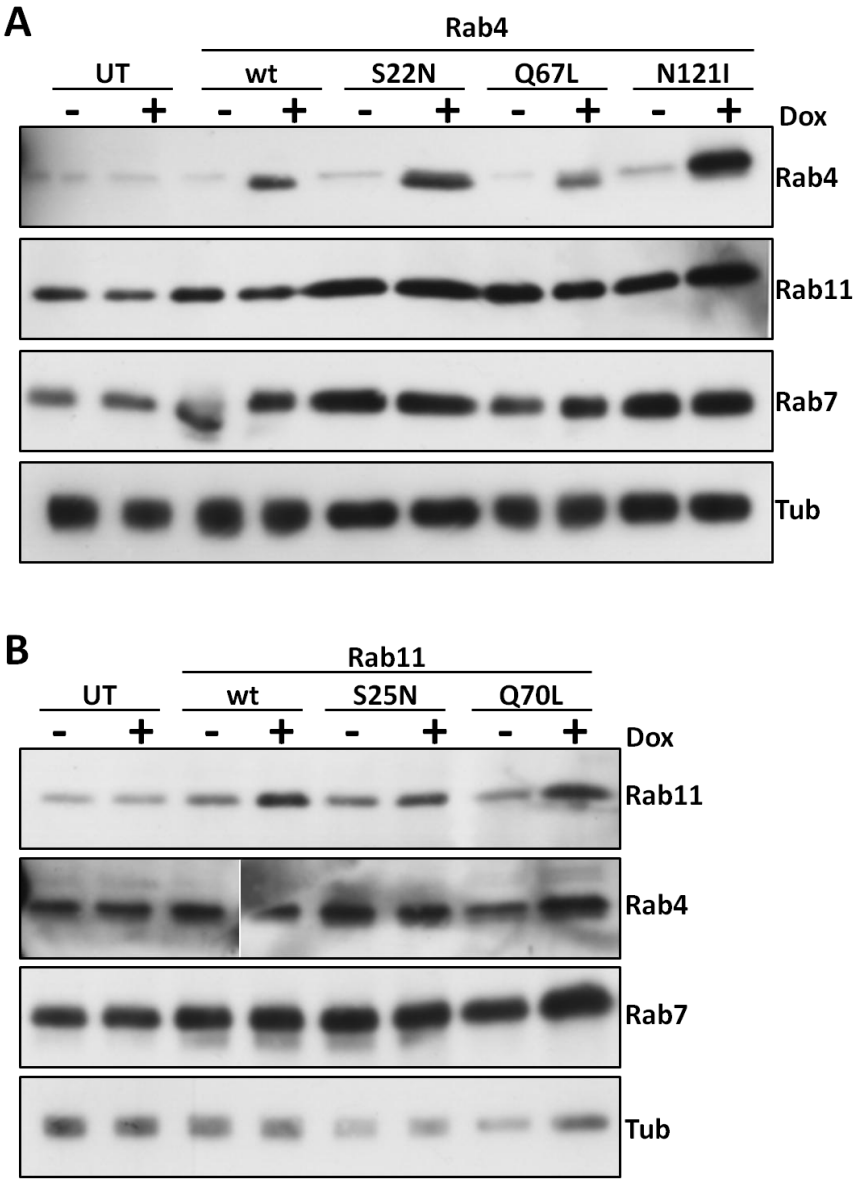

Figure 30. Different Rab proteins are not upregulated in cells expressing wild-type and mutant Rab4 or Rab11.

(A) Comparison of expression levels of Rab4, Rab11, Rab7 and Rab15 in HeLa T-REx cells stably expressing wild-type (wt), dominant negative (S22N, N212I) and constitutively active (Q67L) Rab4, either induced for 24 h with doxycycline or left uninduced. Untransfected cells (UT) were used as control and tubulin (Tub) as a loading control.
(B) Comparison of expression levels of Rab4, Rab11, Rab7 and Rab15 in HeLa T-REx cells stably expressing wild-type (wt), dominant negative (S25N) and constitutively active (Q70L) Rab11, either induced for 24 h with doxycycline or left uninduced. Untransfected cells (UT) were used as control and tubulin (Tub) as a loading control. Lanes separated by white lines are from the same blot.

Discussion

Endosomes are a major sorting station in the late secretory system. They receive cargo molecules from the plasma membrane and the TGN. At endosomes these cargo molecules are then sorted either to the plasma membrane, the late endosomes and subsequently the MVBs and lysosomes, and the TGN (Grant and Donaldson, 2009). To ensure specific and efficient transport of cargo molecules from endosomes to their respective target compartment many different proteins are involved. An important set of regulatory proteins in the secretory system are the Rab GTPases (Stenmark, 2009). Rab4 has been implicated to play a central role in sorting cargo of molecules from early sorting endosomes towards the plasma membrane. Several publications have attributed Rab4 an involvement in recycling of the TfR, but its exact function remains elusive. Furthermore, these studies do not entirely agree on the observed effect on TfR recycling in cells either overexpressing a dominant negative Rab4 mutant or in knockdown cells (Deneka et al., 2003; McCaffrey et al., 2001; van der Sluijs et al., 1992; Yamamoto et al., 2010).

Rab11, on the other hand, has been implicated to play an important role in recycling from recycling endosomes to the plasma membrane. Rab11 could thereby play a role in cargo transport from early sorting endosomes to recycling endosomes, from recycling endosomes to the plasma membrane, or both. The implication of Rab11 in cargo transport from early sorting to recycling endosomes stems mainly from evidence concerning the Rab11 effectors Rab11-FIP3, Rab11-FIP5 and Rab11-FIP2, that have been shown to be involved in correct perinuclear positioning of recycling endosomes, transport of TfR from early sorting endosomes to recycling endosomes, and interaction with the Rab5 and Rab4 effector Rabenosyn-5 (Inoue et al., 2008; Naslavsky et al., 2006; Schonteich et al., 2008). A direct involvement of Rab11 in Tf recycling has also been suggested (Ren et al., 1998). Regulation of transport by Rab11 from recycling endosomes to the plasma membrane has been indicated for the TfR, MHC class I, β1-integrin, β2-adrenergic receptor, and TGF-β (Mitchell et al., 2004; Moore et al., 2004; Powelka et al., 2004; Ullrich et al., 1996; Weigert et al., 2004). Interestingly, in polarized cells Rab11 was shown not to be important for basolateral TfR recycling, but for apical IgA and E-cadherin recycling (Lapierre et al., 2001; Lock and Stow, 2005).

In the present study we used a novel *in vivo* recycling assay to investigate the role of Rab4 and Rab11 on TfR recycling more closely and an *in vitro* carrier formation assay to investigate the role of Rab4, Rab11, and Rab14 on the formation of TfR and EGFR containing endosomal carriers.

In vitro Formation of Transport Carriers

In our lab, an *in vitro* assay has been developed to study the formation of transport carriers at endosomes. In this assay, carrier formation is reconstituted in permeabilzed cells by addition of an energy source and exogenous cytosol. In order to determine whether a protein plays a role in carrier formation, the cytosol is modified by adding or removing this protein, or a mutant form of it. The need for cytosol modification may create challenges due to lack of suitable antibodies for immunodepletion or difficulties in purifying the protein of interest. Cell culture derived cytosol offers new modification possibilities, since proteins can be overexpressed or their expression silenced in the cells prior to cytosol production. To test whether production of cell culture derived cytosol was feasible, cytosol was extracted from Cos-1 and HeLa T-REx cells. Cytosol could be extracted from both cell lines in sufficient quantities and concentrations to be useful in the *in vitro* carrier formation assay. The protein concentration of cell culture derived cytosol was approximately 5 µg/µl to 20 µg/µl and is comparable to that of calf brain cytosol, which lies between 15 µg/µl to 30 µg/µl. The fluctuations in protein concentration between individual cytosol preparations can be explained by the difficulty to resuspend the cells in an equal amount of transport buffer after they have been harvested. This leads to variations in the dilution of the cytosol with transport buffer. The fluctuation in total protein amount isolated between individual cytosol preparations can be explained by differences in the amount of grown cells and by losses during harvesting, resuspension, and homogenization. The variations in cytosol quantity and concentration between HeLa T-REx cells and Cos-1 cells can be explained by differences in cell morphology and the fact that HeLa T-REx cells do not grow to full density on culture dishes.

Our laboratory has previously used polarized MDCK cells in the *in vitro* carrier formation assay. However, MDCK cells are polarized, while calf brain cytosol predominantly contains the components of the non-polarized recycling machinery. Therefore non-polarized BHK cells were used in this thesis. Because the asialoglycoprotein receptor is only endogenously expressed in hepatic cells, the ubiquitously expressed TfR was used as cargo molecule to follow carrier formation. Furthermore, the BHK cells that were used also overexpressed the EGFR, a ligand dependent signaling receptor. To establish whether BHK cells were suitable for the assay, cytosol and AP-1 dependence were tested, since the formation of asialoglycoprotein receptor-containing endosome-derived carriers was blocked upon AP-1 depletion of the cytosol in MDCK cells (Pagano et al., 2004). We could show that *in vitro* carrier formation in BHK cells is dependent on cytosol and AP-1. This finding indicated an involvement of AP-1/clathrin coats in the formation of EGFR containing carriers at endosomes. We also showed that cell culture derived cytosol promotes carrier formation to a similar extent as calf brain cytosol, making it a valuable alternative to brain derived cytosol.

We were interested in the role of Rab4, Rab11 and Rab14 in carrier formation at endosomes. In order to test these Rabs in the *in vitro* assay, Cos-1 cells were transfected with wild-type, dominant negative and constitutively active Rab4, Rab11, and Rab14 and the cytosol was prepared. Additionally, HeLa T-REx cells were transfected with the same Rab proteins and their respective mutants, and with plasmids containing shRNA directed against Rab4 and Rab11, respectively. Clones stably expressing these proteins or shRNA under an inducible promoter were selected. The advantage of using Cos-1 cells is a strong overexpression in the transfected minority of cells. As a result, most of the cytosol originates from untransfected and thus unaltered cytosol, an effect that lowers the possibility of indirect effects caused by adaptation of transfected cells. Disadvantages are varying expression levels between cytosol preparations and a need for copious amounts of plasmid DNA or siRNA, and transfections reagent. Stable cell lines offer the advantage of similar expression levels between individual cytosol preparations of the same cell line.

There was a concern that much of the Rab proteins, which reversibly associate with membrane via a prenyl group, might be lost during cytosol production. Indeed, in CHO cells 75% of Rab4 had been found to associate with membranes (Van Der Sluijs et al., 1991). Overexpressed wild-type or constitutively active mutants might even associate with membranes more profoundly. Dominant negative Rab mutants are thought to overrule the endogenous protein by sequestering the GEF, which could also lead to membrane association of the Rab, should the GEF be a membrane protein (Li et al., 1994). To address this issue, cytosol from untransfected Cos-1 cells was compared to cytosol from Cos-1 cells expressing wild-type, dominant negative, and constitutively active Rab4. A several fold greater level of Rab4 was detected for all Rab4 constructs in comparison to untransfected cells, indicating that Rab loss during cytosol production is not a problem. We also looked at the membranes of HeLa T-REx cells expressing wild-type, dominant negative, and constitutively active Rab4. Indeed, the membrane fractions of cells overexpressing Rab4 or mutants contained more Rab4 than in untransfected cells. However, Rab4 levels were also several folds greater in the cytosol fractions of transfected versus untransfected cells. Finally, we tested whether the Rab4 effector Rabaptin-5 was depleted from the cytosol by being recruited to the membranes by the large amounts of Rab4 and mutants in transfected cells. Rabaptin-5 was found to be present in the cytosol from cells overexpressing Rab4 and mutants. An increase in membrane associated Rabaptin-5 was not evident, suggesting that effector depletion is also not an issue of cell culture cytosol production.

Our laboratory has shown that depletion of Rab4 from calf brain cytosol leads to an inhibition of the formation of endosomal carriers containing the asialoglycoprotein receptor H1 in MDCK cells (Pagano et al., 2004). To investigate whether Rab4 also plays a role in the formation of endosomal carriers containing TfR and EGFR in BHK cells, we used cell culture derived cytosol in our *in vitro* assay.

Indeed, the dominant negative Rab4 mutant S22N and the constitutively active mutant Q67L both had a partial inhibitory effect on the formation of TfR containing carriers, while wild-type Rab4 had no influence. Surprisingly, the other dominant negative Rab4 mutant N121I had no significant effect on the formation of endosomal carriers. Indeed, there is a difference between the two mutants, S22N is able to bind GDP but not GTP, N121I on the other hand has a reduced guanine nucleotide binding affinity (Cormont et al., 1996; Gerez et al., 2000). It is tempting to suggest, that the inability of N121I to bind a guanine nucleotide renders it unable to influence the specific step of carrier formation. These two types of mutations have previously been observed to not always produce the same phenotype. For example the Rab1b S22N mutation caused partial Golgi disruption, whereas the N121I mutant completely disrupted the Golgi structure, caused relocation of Golgi resident proteins to the ER and dissociation of β-COP from membranes (Alvarez et al., 2003). Furthermore, the Ras mutant D119N was found to act both dominant negative and constitutively active, depending on its concentration in the cell (Cool et al., 1999). Ras D119N binds and sequesters the GEF, thereby being dominant negative. As soon as there is no more unbound GEF, this mutant, which is able to bind GTP without GEF activity, will now be able to become active. This is possible, because D119N has a decreased nucleotide affinity, which allows it to bind GTP, while Ras S17N has strongly decreased GTP affinity, similar to the Rab4 N121I and S22N mutants (Farnsworth and Feig, 1991; Powers et al., 1989; Stacey et al., 1991). The effect of Rab4 and mutants on EGFR containing carriers was similar, with the exception of the constitutively active mutant that did not impair carrier formation, indicating that TfR and EGFR may be transported within different carriers from endosomes to the plasma membrane.

Rab11 is generally assumed to be the major regulator of the slow recycling pathway (Ren et al., 1998; Ullrich et al., 1996). Our laboratory has previously wanted to investigate the role of Rab11 on carrier formation with our *in vitro* assay. However, this experiment has been delayed by the lack of Rab11 antibodies suitable for immunodepletion. Therefore we tested the role of Rab11 by using cell culture derived cytosol from cells expressing wild-type, dominant negative, and constitutively active Rab11. An inhibitory effect on the formation of TfR and EGFR containing endosomal carriers in the presence of dominant negative and constitutively active Rab11 was observed. On the other hand, wild-type Rab11 had no effect on carrier formation. The fact that both the active and the inactive mutant had in inhibitory effect on carrier formation is consistent with two previous studies that showed both of these mutants to negatively affect the recycling rate of the TfR (Ren et al., 1998; Ullrich et al., 1996). Interestingly, the inhibitory effect on TfR containing carrier formation was only partial, indicating that the TfR is transported via more than one carrier and not solely dependent on Rab11. Furthermore, since we observe carrier formation of cargo molecules that have been internalized for 10 min, it is possible that we observe transport from early sorting to recycling endosomes and not transport from

recycling endosomes to the plasma membrane. Indeed, Rab11 has been implicated to be an important factor in this trafficking step, as described above.

Rab14 was shown to localize to Rab4 and Rab11 positive endosomes and to be able to interact with the Rab4 effector Rabip4 and the class 1 Rab11-FIPs (Kelly et al., 2010; Proikas-Cezanne et al., 2006; Yamamoto et al., 2010). Whether Rab14 is also involved in TfR recycling is not entirely clear. An early study could not show an influence of Rab14 on recycling, while a more recent study suggests that Rab14 is required for efficient recycling of Tf to the cell surface (Junutula et al., 2004; Yamamoto et al., 2010). Because of this, we also tested wild-type, dominant negative and constitutively active Rab14 in the *in vitro* carrier formation assay. We could not observe any influence of Rab14 or Rab14 mutants on TfR or EGFR containing carrier formation. These findings suggest that Rab14 is not directly involved in endosomal carrier formation. The presence of Rab14 at endosomes can also be explained with a function in TGN-to-endosome transport. For example, Rab14 has been shown to bind the kinesin motor protein KIF16B and the Rab14/KIF16B complex has been shown to be required for FGFR transport from the TGN to endosomes (Ueno et al., 2011).

In Vivo Recycling of the Transferrin Receptor

As mentioned above, the role of Rab4 in recycling is somewhat ambiguous. Rab4 was observed to alter the steady state distribution of the TfR, an indication for defects in endocytosis and recycling (van der Sluijs et al., 1992). However, in the same study, neither Tf uptake nor Tf return to the plasma membrane were affected in CHO cells overexpressing wild-type or dominant negative Rab4. Similarly, expression of wild-type, dominant negative, and constitutively active Rab4 in MDCK cells did not alter basolateral Tf endocytosis or recycling (Mohrmann et al., 2002b). In contrast to these findings, it was reported that Tf recycling was strongly inhibited in HeLa cells expressing dominant negative Rab4 and slightly increased in cells expressing wild-type and constitutively active Rab4 (data not shown in McCaffrey et al., 2001). Furthermore, RNAi mediated knockdown of Rab4 was reported to enhance the recycling rate of Tf in HeLa cells in one study and to slow the recycling rate in another (Deneka et al., 2003; Yamamoto et al., 2010).

Because of these ambiguities, we examined the role of Rab4 on TfR recycling again. To test this we developed a novel endocytosis and recycling assay using fluorescently labeled Tf and automated image acquisition in combination with automated image analysis. The advantages of this method are the large number of cells that can be quantified and the possibility to monitor cell morphology. As a proof of concept we performed the recycling assay in HeLa cells and found Tf recycling rates to be in the range of previously established data (Ciechanover et al., 1983). Furthermore, we could show an

almost complete block of Tf recycling in the presence of Monensin, an ionophore antibiotic that is known to inhibit Tf recycling (Stein et al., 1984). Additionally, we could show an almost complete block of Tf endocytosis in the presence of the dynamin inhibitor Dynasore (Macia et al., 2006).

To test the influence of Rab4 and mutants of Tf recycling we wanted to use our HeLa T-REx cells stably expressing these proteins and their mutants under an inducible promoter. However, the HeLa T-REx cells proved to be very sensitive to any kind of stress, which is observable as a strong morphological reaction of the cells contracting and rounding up. A close examination of the individual steps of the recycling assay showed that warming the cells from 4°C to 37°C had an especially strong effect on their morphology. This issue also affected the recycling assay, since the mean intensity (average pixel intensity within a cell) was measured to quantify the intensity of fluorescent transferrin. Since round cells are smaller than normal cells, the same quantity of fluorescent Tf is packed denser in rounded cells compared to normal cells. Because the cells used to measure the starting intensity of internalized fluorescent Tf (the 100% or 0 min time point) were not warmed to 37°C and thus did not round up, cells that were only allowed to recycle for a few minutes appeared to contain more fluorescent Tf. This effect can be reversed by measuring the sum of all pixel intensities within a cell (total intensity per cell), since the rounded cells are smaller and thus have fewer pixels when compared to normal cells. However, cell contraction may also lead to other cellular reactions, altering the experimental outcome. To circumvent this problem the assay protocol needed to be modified in a way to avoid temperature shifts.

It should also be noted, that a variability of the recycling rate was observed between individual experiments. The reason for this inter-assay variability is not clear. It is therefore essential to compare different conditions in parallel on the same assay plate to make sure that differences observed between induced and uninduced, or transfected and untransfected cells are not due to inter-assay variability. Furthermore, one should keep fluctuation of individual experiments in mind when comparing results from individual assay plates with each other.

Following assay optimization, we tested our HeLa T-REx cells expressing dominant negative (S22N and N121I) and constitutively active (Q67L) Rab4 mutants and compared induced with uninduced cells. The recycling rate in Rab4 S22N expressing cells was investigated first, and recycling was stopped every 2 min for 20 min. No significant effect on recycling could be observed, indicating that Rab4 is not required for efficient Tf transport from endosomes to the plasma membrane. We also tested cells expressing Rab4 Q67L and N121I but could not observe changes in the rate of Tf recycling. Next we tested the effect of Rab4 knockdown on Tf recycling. For this experiment we decided to use HeLa cells and perform a siRNA mediated knockdown using a pool of four different siRNAs. As determined by semi-quantitative immunoblot analysis, knockdown of Rab4 was

approximately 90%. However, no changes in the Tf recycling rate were observed, again indicating no requirement for Rab4 in efficient Tf transport from endosomes to the plasma membrane.

Since we did not observe an influence of Rab4 on Tf recycling, it is possible that Rab4 is rather required for recycling of other cargo molecules. Indeed, it was shown that the β-2 adrenergic receptor recycles in a Rab4 depended fashion (Yudowski et al., 2009). Furthermore, a recent study has shown Rab4 to co-localize with the β-2 adrenergic receptor in tubules protruding from endosomes while the TfR localized to tubules devoid of the β-2 adrenergic receptor (Temkin et al., 2011). These findings indicate that Rab4 containing tubules could also be largely devoid of the TfR and it will therefore be very interesting to directly compare Rab4 and TfR localization on endosomes with the same high resolution that was used in the above study.

Rab11 was previously shown to moderately impair Tf recycling, therefore we also tested the role of Rab11 with our recycling assay (Ren et al., 1998; Ullrich et al., 1996). Surprisingly, neither wild-type, nor dominant negative, nor constitutively active Rab11 had a notable effect on Tf recycling. Furthermore, shRNA mediated knockdown of Rab11 did also not impair Tf recycling. To exclude that this was a HeLa T-REx specific finding, we then transiently transfected HeLa cells with GFP coupled dominant negative Rab11 and measured Tf recycling in transfected compared to untransfected cells. Again, we could not observe an effect on Tf recycling. However, it should be noted, that the transfection efficiency was poor with only around 100 transfected cells per time point. Why we could not reproduce the effect of Rab11 on Tf recycling that other studies have shown can only in part be explained by differences in the methodology. Another possibility may be cell type specific differences.

Similar to Rab4, Rab11 has also been shown to be required for recycling of other cargo molecules, like β1 integrin, β2 adrenergic receptor, TGF-β and E-cadherin (Lock and Stow, 2005; Mitchell et al., 2004; Moore et al., 2004; Powelka et al., 2004). Furthermore, Rab11 interacts with myosin Vb, a motor protein required for IgA recycling. Interestingly, Tf recycling is also impaired when a GFP-myosin Vb tail chimera is overexpressed in HeLa cells but not in MDCK cells. These findings indicate that Rab11 may not be directly required for efficient Tf recycling, but possibly rather regulates trafficking of different cargo molecules.

Conclusions

Our results show an involvement of Rab4 and Rab11 in endosomal carrier formation *in vitro*, while overall recycling *in vivo* was not dependent on the activity of these two Rab proteins. Since the TfR is recycled constitutively and is transported via separate pathways through the endosomal system, it is possible that it is also packaged into different carriers (Grant and Donaldson, 2009). The impairment of only one type of carrier may then not greatly affect the overall recycling rate of the TfR. Pathways may also adjust to the alterations induced by overexpression of Rab proteins and mutants. The fact that we can observe an effect on carrier formation in our *in vitro* assay indicates that the TfR is trafficked in Rab4 and in Rab11 dependent carriers. However, the observed impairment of carrier formation is only partial, making it tempting to suggest that the TfR is partially trafficked within the Rab4 and the Rab11 dependent pathways but is not confined to one of the two. Furthermore, the TfR may even be transported through additional Rab4 and Rab11 independent recycling pathways. It should also be kept in mind that *in vivo* experiments are performed 24 h post induction or transfections, in the case of RNAi mediated silencing even longer, leaving time for the cells to adapt to changes. In contrast, our *in vitro* experiments offer cells little chance for adaptation. It is therefore possible that a phenotype is less prominent in an *in vivo* experiment than in an *in vitro* experiment. For example, the TfR may be efficiently trafficked through a compensatory pathway it would not be transported in under normal conditions, concealing the function of a protein, while the function of this protein would become evident by looking at a specific step, instead of a complete pathway. An involvement of Rab4 in endosomal carrier formation is also supported by the fact that the Rab4 effector Rabaptin-5 binds AP-1, an important adaptor of clathrin mediated vesicle formation (de Renzis et al., 2002; Deneka et al., 2003; Vitale et al., 1998).

Rab proteins can fulfill several diverse functions. For example, Rab5, one of the best studies Rabs, recruits its effector PI3 kinase to endocytic vesicles (Shin et al., 2005). PI3 kinase is involved in changing the lipid composition of the vesicle from to PtdIns(4,5)P_2 to PtdIns(3)P. This lipid turnover is in part responsible for the uncoating of endocytic vesicles, since AP-2 requires PtdIns(4,5)P_2 to be recruited to membrane (Honing et al., 2005; Semerdjieva et al., 2008). Furthermore, PtdIns(3)P is also required to recruit the Rab5 effector EEA1 to endocytic vesicles (Callaghan et al., 1999; Lawe et al., 2000). By observing a complete pathway like recycling of TfR, it is therefore not possible to determine the mechanism of the Rab function. To investigate the mechanistic function of Rab proteins it is necessary to dissect the recycling pathway and to look at individual steps like carrier formation, as we do with our *in vitro* assay. Further research will be necessary to determine the mechanistic functions of Rab4 and Rab11, and in general the processes of endocytic recycling more accurately.

Outlook

Further studies will be needed for a more precise characterization of TfR recycling and the role of Rab4 and Rab11 in particular, and of the processes of endocytic recycling in general. Our results suggest that the TfR is at least partially transported in a Rab4 and Rab11 dependent fashion, albeit without a strict requirement for either of the two. Therefore it will be very interesting to test whether TfR recycling is affected when both the Rab4 and the Rab11 pathways are simultaneously impaired. This can be achieved by either co-transfecting dominant negative or other mutants of Rab4 and Rab11, or by double knockdown of the two proteins. Furthermore, the influence on TfR recycling can also be tested for other candidate Rab proteins that have been implicated in recycling, like Rab15 and Rab35, and combinations thereof. Endocytosis of the TfR is highly dependent on dynamin, as published before and shown in this thesis (Macia et al., 2006). To test whether dynamin also plays a role in TfR transport from endosomes to the plasma membrane, the potent dynamin inhibitor dynasore can be used in the recycling assay.

Since the TfR is transported via more than one pathway within the endosomal system and possibly could be packaged into more than one distinct carrier, it may be helpful to follow other recycling receptors like signaling receptors that are transported in a more regulated fashion. For example, a method to observe recycling of the β-2 adrenergic receptor has already been established and can possibly be adapted to work in our own recycling assay (Yudowski et al., 2009). A recent study has shown Rab4 to co-localize with the β-2 adrenergic receptor in tubules protruding from endosomes while the TfR localized to different tubules (Temkin et al., 2011). High resolution microscopy may be used to co-localize the TfR with different Rabs or other proteins of the recycling machinery to elucidate the mechanism by which the TfR is transported.

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Figures 10, 12, 15 and 17 partially include data of experiments performed by Dr. Barry Shortt.

Figure 10 partially includes data of experiments performed by Nicole Beuret.

Curriculum Vitae

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