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THE ROLE OF MEMBRANE DOMAINS IN PROTEIN AND LIPID SORTING DURING ENDOCYTIC TRAFFIC

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A

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Dedication

I want to dedicate this work to my wife Cara and children Benjamin and Samuel, as well as my family and mentors that have supported me along the way.

Acknowledgments

First and foremost, I would like to thank Ilya and Kandice Leventhal, my two mentors through this whole journey. Not only were they great teachers that showed me how to do good science, but they also believed in me and always trusted that I could achieve this, even when I didn't. Their support inside and out of the lab was critical in my success.

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THE ROLE OF MEMBRANE DOMAINS IN PROTEIN AND LIPID SORTING DURING ENDOCYTIC TRAFFIC

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The lipid and protein composition of the plasma membrane (PM) must be tightly controlled to maintain cellular functionality, despite constant, rapid endocytosis. Because *de novo* synthesis of proteins and lipids is energetically costly, the cell depends on active recycling to return endocytosed membrane components back to the PM. For most proteins, the mechanisms and pathways of their PM retention remain unknown. The work presented here shows that association with ordered membrane microdomains is fully sufficient for PM recycling and that abrogation of raft partitioning leads to their degradation in lysosomes. These findings support a model wherein ordered membrane domains mediate PM recycling of membrane components from the endosomal system. The next step was to identify the pathways and molecular players responsible for raft-mediated recycling. Using orthogonal transmembrane protein probes for raft and non-raft domains, I identified and validated cellular machinery that act as trafficking mediators specific for recycling of raft-associated proteins to the PM. This raft-mediated pathway is not dependent on the classical recycling pathways defined by Rab4 and Rab11, but instead represents a novel route for PM recycling of raft-preferring cargo from late endosomes. I implicate Rab3 as a central regulator of this pathway and show that the Rab3 family is essential for PM homeostasis, as abrogation of all four members of the Rab3 family disrupts PM recycling of lipid raft associated proteins. The findings reveal a fundamental role for raft microdomains in endocytic sorting and recycling and support a novel role for Rab3 as a central regulator of a previously unrecognized mechanism for PM and endosome homeostasis.

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BafA1: bafilomycin A1

BFA: brefeldin A

EE: early endosome

EGFR: epidermal growth factor receptor

ER: endoplasmic reticulum

GPCR: G-protein coupled receptor

GPMV: giant plasma membrane vesicle

LE: late endosome

PM: plasma membrane

SBP: streptavidin binding peptide

RUSH: retention using selective hooks

TfR: transferrin receptor

TMD: transmembrane domain

Chapter 1

Introduction

This chapter is based upon “Diaz-Rohrer B, Levental KR, Levental I: **Rafting through traffic: Membrane domains in cellular logistics**. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 2014, **1838**(12):3003-3013.”

1.1 Cell Membranes

The membranes in the cell are composed of proteins and lipids generally organized in a lipid bilayer described by Singer and Nicolson's fluid mosaic model (Singer & Nicolson, 1972). The membrane is in a fluid state, which allows proteins and lipids to freely diffuse laterally. There have been many additions to this model, but the major principles still hold.

The composition of a membrane is fundamentally important for its function. The types of lipids in a membrane can determine physical properties like rigidity, curvature, thickness and viscosity. The types of proteins (i.e. receptors, channels, enzymes, etc.) recruited to the different cellular membranes are essential for the functions of various organelles. The proteins of the membrane can be attached to one of the bilayer leaflets or cross the bilayer, both of which contribute significantly to membrane structure (Steck, 1974; Stone, Shelby, & Veatch, 2017).

Eukaryotic cells are organized into distinct cellular compartments that are spatially segregated and functionally different. The majority of these organelles are delimited by a membrane composed of proteins and lipids. This membrane is both the barrier and interface between the organelle and the rest of the cell. In order to maintain the function and identity of each one of these organelles, the composition of their surrounding membranes must be tightly regulated. Despite physical and functional organelle separation, there is constant communication between them. Organelle communication is necessary for the cell's functionality and survival. At any point in time, there are a vast number of distinct vesicles trafficking proteins and lipids from one compartment of the cell to another. Therefore, accurate sorting and recycling of membrane components is necessary for life.

1.1.1 Plasma Membrane

The PM serves as a physical barrier and a communication interface of the cell. The membrane must be impermeable to maintain the intracellular composition as well as the cells shape and volume, at the same time allowing passage of small molecules and ions necessary for the cell. This selective permeability is achieved by proteins that function as transporters and channels (Keren, 2011). As the communication hub with the extracellular environment, the PM is also responsible for sensing extracellular cues and acting on those cues. Finally, the PM plays a major role in trafficking pathways, including both secretion and endocytosis, which have to be synchronized to maintain cell size and shape. An increase in endocytosis can trigger exocytosis to maintain the membrane (Gauthier, Fardin, Roca-Cusachs, & Sheetz, 2011; Masters, Pontes, Viasnoff, Li, & Gauthier, 2013). PM homeostasis is of central importance to the cell, yet it is a highly dynamic organelle with an estimated turnover time of ~20 min (Thilo & Vogel, 1980). Because of the time constrains and high energy requirement of *de novo* synthesis of proteins and lipids, the cell depends on recycling of endocytosed proteins and lipids back to the PM to maintain its structure and function. However, the process of how the cell determines which components to recycle is not clear.

1.1.2 Protein and lipid sorting

The localization of a protein in a cell determines which partners it interacts with and allows the protein to be integrated in the biological network of the cell. There are many instances in which the same protein can act in different ways depending on its localization. For example, a protein in the cytosol can be inhibited by interaction with another molecule, but if the same protein is translocated to the nucleus it can bind to a

partner and be activated. When a protein is taken out of its native environment it can result in dysregulation of its activity. Aberrant protein localization has been linked to several diseases including metabolic, cardiovascular and neurodegenerative diseases (Hung & Link, 2011). Many proteins are sorted by specific protein-protein interactions, including several known cytosolic signals for adapter- and coat-mediated sorting between cellular organelles (Bonifacino & Traub, 2003; Mellman & Nelson, 2008)

1.2 Membrane Trafficking

Membrane trafficking takes place in small vesicles that require specific machinery in order to bud, separate from the originating organelle (in a process known as fission), and integrate into the proper destination organelle (known as targeting and fusion). Each of these steps is orchestrated by dedicated protein machinery. The identification and characterization of this protein machinery has been a major area of research for several decades. These distinct classes of proteins are often classified by their function.

1.2.1 Coat Proteins

Coat proteins assemble at the membrane and help concentrate cargo while at the same time mediating vesicle formation. There are three well studied coat proteins. Clathrin mediates endocytosis from the PM as well as vesicle formation from the Golgi to lysosomes (Goldstein, Anderson, & Brown, 1979). The Coat Protein complexes, COPII and COPI act in opposing directions to deliver cargo from the endoplasmic reticulum (ER) to the Golgi and *vice versa*, respectively (Barlowe et al., 1994; Orci, Glick, & Rothman, 1986). Cavins and caveolins from a two protein complex that also

plays a role in PM endocytosis (Rothberg et al., 1992). Other vesicle forming machinery like the ESCRT pathway (Raiborg & Stenmark, 2009) or clathrin-independent endocytic pathways do not utilize coat proteins, but rather rely on other machinery to create membrane deformation (Kirkham & Parton, 2005; Sabharanjak, Sharma, Parton, & Mayor, 2002). The various endocytic pathways, key protein machineries, and some known cargoes are summarized in Table 1.

1.2.2 Fission Proteins

Once a vesicle is formed after having selected a set of proteins and lipids to be trafficked, it needs to detach from its originating organelle. The most widely studied protein that acts in the process of membrane scission is dynamin, a GTPase that binds at the neck of a budded vesicle and fuses two lipid bilayers together to pinch off a vesicle (van der Bliek et al., 1993). Other proteins that play a role in vesicle scission are the BIN/Amphiphysin/Rvs (BAR) domain protein family. The BAR proteins bind to the membrane, producing membrane curvature which can either promote or inhibit of scission (David, Solimena, & De Camilli, 1994). The formed vesicles are trafficked along microtubules or the actin network by various protein motors, including kinesin, dynein (Hirokawa, 1998), and myosin(Wang et al., 2008). The force that the motors exert on the vesicles by pulling on them can also aid in vesicle scission. And even actin filaments may exert forces at budding necks that aid vesicle scission (Ceridono et al., 2011; Khandelwal, Ruiz, & Apodaca, 2010).

Table 1. Endocytic pathways and their cargo

Endocytic Pathway	Clathrin Coated Pits	Caveola	CLIC/GEEC	ARF6 Dependent	Flotillin
Key Proteins	Clathrin (Pearse, 1976), Epsin (Di Fiore, Polo, & Hofmann, 2003), Intersectin (Yamabhai et al., 1998), Dynamin (van der Bliek et al., 1993), Arf6 (Tanabe et al., 2005), PKC (Robinson et al., 1993), Rac1 (Lamaze, Chuang, Terlecky, Bokoch, & Schmid, 1996), cdc42 (Yang, Lo, Dispenza, & Cerione, 2001), RhoA (Lamaze et al., 1996)	Caveolins (Monier et al., 1995; Rothberg et al., 1992), PKC (Sharma et al., 2004), SRC (Sharma et al., 2004), cdc42 (Klein et al., 2009), Intersectin (Klein et al., 2009), Dynamin(Oh, McIntosh, & Schnitzer, 1998)	RhoA (Lamaze et al., 2001), GRAF (R. Lundmark et al., 2008), cdc42 (Sabharanjak et al., 2002), Arf1 (Lundmark, Doherty, Vallis, Peter, & McMahon, 2008), cortactin (Sauvonnet, Dujeancourt, & Dautry-Varsat, 2005), Arf6 (Richard Lundmark et al., 2008)	Arf6 (Naslavsky, Weigert, & Donaldson, 2004)	Flotillin (Glebov, Bright, & Nichols, 2006)
Known Cargo	GPCR (Wolfe & Trejo, 2007) Transferrin Receptor (Di Fiore et al., 2003) Anthrax Toxin (Abrami, Liu, Cosson, Leppla, & van der Goot, 2003), Cadherin (Bonazzi, Veiga, Pizarro-Cerda, & Cossart, 2008), LDL (Maurer & Cooper, 2006), Influenza (van der Bliek et al., 1993)	GP60 (Minshall et al., 2000), CTX (Anderson, Chen, & Norkin, 1996), SV40 (Cheng, Singh, Marks, & Pagano, 2006), Cadherin (Bonazzi et al., 2008), GPI-AP (Cheng et al., 2006), LacCer (Puri et al., 2001), IL2 (Lamaze et al., 2001)	IL2, SV40 (Damm et al., 2005), GPI-AP (Damm et al., 2005; Sabharanjak et al., 2002)	MHC I (Naslavsky et al., 2004), CD59	CD59, Proteoglycans (Payne, Jones, Chen, & Zhuang, 2007)
Lipid Rafts Implication?	No(Nichols, 2003)	Yes (Monier et al., 1995; Rothberg et al., 1992) Cav1 binds Cholesterol	Yes (Damm et al., 2005; Sabharanjak et al., 2002) GPI-AP found in lipid rafts	Unclear (Gong et al., 2007)	Unclear

1.2.3 Targeting and fusion proteins

The vesicles newly freed of their source organelle next need to reach the correct destination and fuse with the destination organelle. For this step, there are two main protein families involved, the first one being the Rab family of GTPases, composed of more than 60 proteins. The various Rab proteins reside in different organelles acting as cellular “address labels” (Zerial & McBride, 2001). Rab proteins are present in the trafficking vesicles as well as the target organelles (Pfeffer & Aivazian, 2004), with the double label adding specificity to trafficking events. The second family of proteins are the SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptor) proteins. These proteins form a tetramer composed of a SNARE in the vesicle and a trimer of SNAREs in the target compartment membrane (Rice & Brunger, 1999; Sutton, Fasshauer, Jahn, & Brunger, 1998). The complex formation gives specificity to the fusion event because the pairs for v-SNARE and t-SNARE are specific and not all pairs lead to productive fusion (McNew et al., 2000). The complex also serves to promote fusion by binding two other proteins N-ethylmaleimide sensitive factor (NSF) and soluble NSF associated protein (α-SNAP) that serve to overcome the energy barrier to enable membrane fusion (Sollner et al., 1993). Other vesicles tethers also play a role in vesicle fusion, including golgins in the secretory pathway (Barr & Short, 2003) and early endosome antigen 1 (EEA1) in endocytosis (Christoforidis, McBride, Burgoyne, & Zerial, 1999).

1.3 Lipid rafts in membrane trafficking

Rafts were first implicated in sorting of proteins and lipids as a way to create distinct PM domains in polarized cells; the first clue to this phenomenon was the enrichment of glycosylphosphatidylinositol-anchored proteins (GPI-APs) in trans-Golgi network derived vesicles destined for the apical plasma membrane (Simons & van Meer, 1988). Sorting is the step in which de-mixing of components occurs by separating these components based on a shared characteristic. For coat-mediated transport, the shared characteristic is ability to bind tightly to coats and adapters. For lipid rafts, preferential interactions between various lipids and proteins lead to lateral membrane domains. Lipid raft formation acts to enrich or deplete a domain of a particular component, making it an ideal mechanism to laterally sort bulk components within a membrane. These rafts can then serve as platforms that can be used as origin areas for fission of vesicles, which serve as the communication and transport routes across organelles (Fig. 1).

Lipid microdomains have been previously implicated as a sorting mechanism for proteins in the secretory pathway (Brown & Rose, 1992; Schuck & Simons, 2004; Yoshimori, Keller, Roth, & Simons, 1996) and for endosomal recycling (Gagescu et al., 2000; Lusa et al., 2001). Lipid rafts are enriched in sterols and sphingolipids, which have also been shown to be enriched at the PM (Lange, Swaisgood, Ramos, & Steck, 1989; Orci et al., 1981), and are also enriched in vesicles destined for the PM (Klemm et al., 2009; Surma, Klose, Klemm, Ejsing, & Simons, 2011).

Lipid rafts are small and highly dynamic. They are highly dynamic in two ways: first, rafts themselves can associate and dissociate rapidly and components can freely diffuse into, out or, and within domains. Second, rafts can diffuse laterally within a

membrane (Simons & van Meer, 1988). The capacity to dynamically and selectively recruit proteins and lipids makes rafts an ideal sorting mechanism for membrane trafficking.

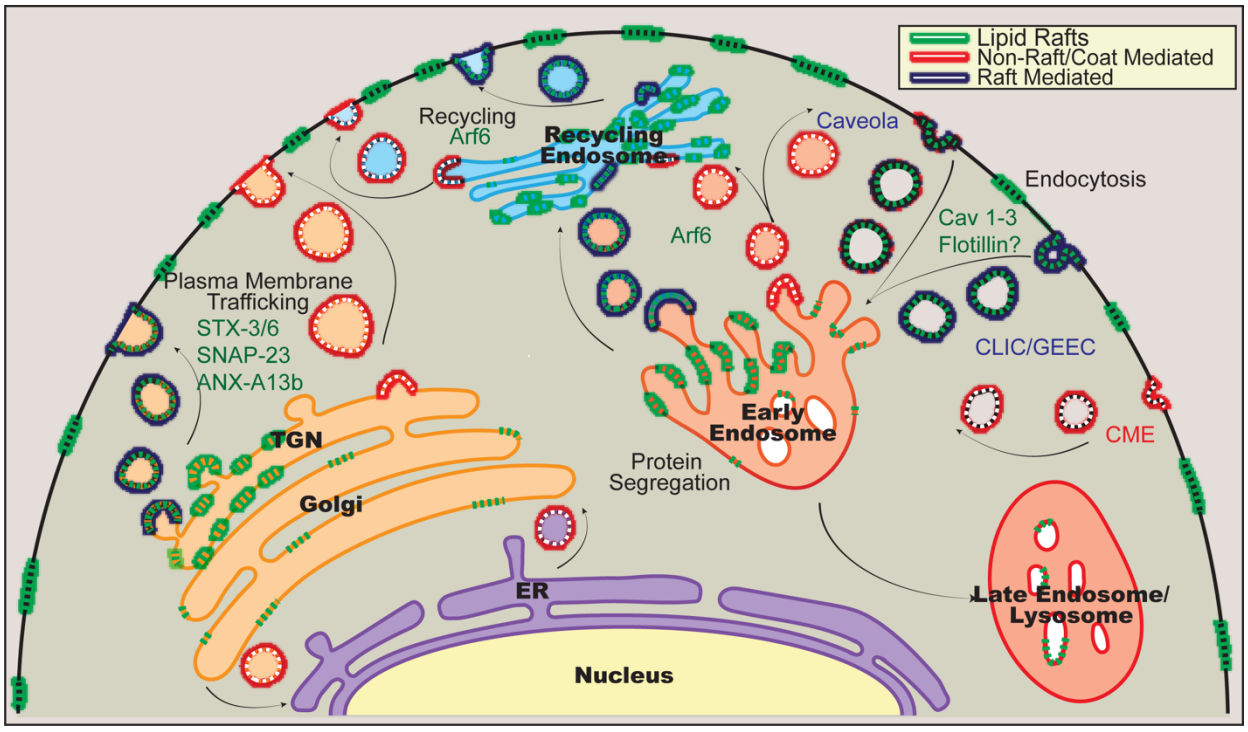


Figure. 1. Involvement of raft domains in membrane traffic. Lateral membrane domains aid in sorting of protein and lipid components between the membranes of subcellular compartments. Membrane rafts (green striped regions) are likely present in the latter stages of the secretory pathway (i.e. the TGN and PM) and early stage of the endosomal pathway (early and recycling endosomes). Rafts recruit components for coordinated exit from a source compartment and traffic to a donor compartment via a raft-enriched vesicular carrier (blue shading around membranes). Such vectoral raft transport includes TGN-to-PM sorting, specific endocytosis at the PM, and recycling from the endosomal systemin the EE and RE. The raft pathway coexists with a number of coat/adapter-mediated pathways (red shading).

1.4 Conclusions

While the endosomal system has been extensively characterized, little is known about how bulk proteins and lipids are sorted. My previous work suggests that partitioning into lipid rafts can target proteins to the PM. These observations imply the existence of a raft-mediated sorting mechanism.

Most of the research to understand how proteins are sorted into diverse subcellular compartments has been done using a specific protein. Even though much has been learned using this approach, it has some inherent limitations. Namely, results are difficult to interpret due to the specificity of protein-protein and protein-lipid interactions, as well as possible protein modifications. Further, the results are difficult to generalize, as it is often unclear which proteins and how many are affected by certain perturbations. In this work I used model raft and non-raft transmembrane domains as probes for raft and non-raft sorting pathways. The probes are composed of a transmembrane domain (TMD) and a fluorescent protein tag, thus these constructs lack any known sorting determinants and have no specific interactions with other proteins. The lack of specific coat/adapter-mediated sorting determinants allows direct investigation of raft-affinity's involvement in protein and lipid trafficking and the characterization of the machinery involved in this process.

Chapter 2

Materials and Methods

2.1 Antibodies

Table 2. Antibodies used.

USE	Item Name *	Vendor	Catalog #
Primary	58K Golgi protein antibody	Fisher Scientific	NC9962137
Primary	actin	Abcam	ab3280
Primary	ARF6 antibody [EPR8357]		ab131261
Primary	Calnexin	Abcam	AB22595
Primary	Caveolin -1	Santa Cruz	sc-894
Primary	EEA1 (C45B10)	Cell Signaling Technology	3288S
Primary	Flotillin-1	Cell Signaling	3253
Primary	FYN	Santa Cruz	sc-16
Primary	GFP		ab290
Primary	Giantin antibody	Abcam	ab24586
Primary	GM130	Cell Signaling Technology	2296
Primary	GOLGA7 antibody	Abcam	ab57381
Primary	LAMP1 (C54H11)	Cell Signaling	3243
Primary	LAMP1 antibody	Abcam	ab24170
Primary	LYN	Santa Cruz	sc-7274
Primary	PAG	Abcam	AB155100
Primary	Rab3	Synaptic Systems	107 003
Primary	Rab11	Cell Signaling	5589
Primary	Rab11 (D4F5) XP	Cell Signaling Technology	5589P
Primary	Rab11a Antibody	Cell Signaling Technology	2413S
Primary	Rab3a	Synaptic Systems	107 011
Primary	Rab5	Cell Signaling	3547
Primary	Rab5 (C8B1)	Cell Signaling	3547P
Primary	RFP	Life Technologies	R10367
Secondary	Amersham ECL Rabbit IgG, HRP	GE	NA934
Secondary	Goat anti-Mouse IgG Alexa Fluor 647	Life Technologies	A-21236
Secondary	Goat anti-Rabbit IgG Alexa Fluor 488	Life Technologies	A-11008
Secondary	Goat anti-Rabbit IgG Alexa Fluor 647	Thermo Fisher Scientific	A27040
Secondary	Mouse IgG HRP Linked Whole Ab	Millipore Sigma	GENA931

2.2 Cell culture and transfection

Human embryonic kidney 293 (HEK-293), epidermoid carcinoma A431 and cervical cancer HeLa cells were grown in Eagle minimum essential medium (EMEM) with 10% fetal bovine serum.

To create stable cell lines expressing LAT_{TMD} and All-Leu TMD constructs, I transfected cells with pEF6-trLAT and pEF6-trAllL plasmids using Lipofectamine 3000. Forty-eight hours after transfection, the cells were selected using 8 µg/ml of blasticidin; for 2 weeks. After that, the cells were maintained in complete media with 2 µg/ml of blasticidin.

2.3 Plasmids and viruses

For the initial screen, a plasmid (pCDNA3.1) expressing the TMD of LAT linked to red fluorescent protein (RFP) was created with EcoRI /BamHI restriction endonuclease sites flanking the TMD coding sequence for easy substitution that allowed the creation of a library of TMD attached to RFP. For the second part, in order to create stable cell lines, the construct was transferred to a pEF6 vector. Green fluorescent protein (GFP) N-terminal tagged Rab4/5/7/9/11 plasmids as well as the GTP- and GDP-bound mutants were obtained from the Michael Davison collection deposited in Addgene. A Rab3A and Rab3B plasmid was purchased from GenScript and used to transfer the Rab3A and Rab3B sequence to an EGFP-N1 plasmid. Site-directed mutagenesis (kit from Agilent) was used to produce the GTP- and GDP-bound mutants.

2.4 K_{raft} Calculation

The plasmids that code for the distinct TMD probes were transfected into HEK-293 cells that were used to produce giant plasma membrane vesicles (GPMV). The coefficient of raft partitioning K_{raft} was obtained by measuring the fluorescence intensity of the protein on the raft domain compared with that of the non-raft domain, which is labeled by a lipid marker. Using GPMV allowed us to calculate a K_{raft} for each protein in a "native" environment, with all the lipids and proteins that are present at the PM. Lipid rafts are small and dynamic, but through the cooling down of GPMVs, the domains coalesce into macroscopic domains that can be easily seen under a regular fluorescence microscope (Fig. 2B).

2.5 High-throughput screening

A library of siRNAs for membrane trafficking proteins that contained 147 different proteins, each one with a pool of four different siRNAs was used. I plated the HEK-293 clonal cell lines expressing LAT_{TMD} and All-Leu in 96-well black plates with an optical-grade film bottom. The cells were then transfected with siRNA pools using Lipofectamine 3000, and 48 hours after transfection, the cells were fixed. The PM was labeled using DiD, and the nucleus was labeled with DAPI (4′,6-diamidino-2-phenylindole). Each plate was imaged using the Nikon A1R high content imaging platform. For each well, six fields were imaged; the fields were selected at random within the well excluding the center and edge. The images were then analyzed with CellProfiler to determine the fluorescence of the probe in the whole cell and at the PM. A ratio of the intensity at the PM over the intensity of the whole cell was calculated for each cell resulting on the fraction of the probe localized at the PM. Several negative

controls were used: a non-transfected control, a non-targeting siRNA, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) siRNA. The cells transfected with the targeted siRNA were compared with the negative controls using two-way analysis of variance. Each siRNA was tested in triplicate, and a hit was determined if the same siRNA significantly differed from the negative controls in two or more of the replicates

Table 3. List of siRNA for high throughput screen

Plate Well	Gene symbol	Gene accession	Duplex catalog number	Sequence 1	Sequence 2	Sequence 3	Sequence 4
1 A02	ADAM10	NM_001110	J-004503-06	CAUCUGACCC UAAACCAAA	CAAGGGAAGG AAUAUGUAA	CGAGAGAGUU AUCAAAUGG	GAACUAUGGG UCUCAUGUA
1 A03	AP2A1	NM_130787	J-012492-05	CCGAUGAGUU GCUGAAUAA	GGAGCAAUGC CAAGCAGAU	GCAAGAAGAA CCCAGAUGA	CCAAGAAGGU GCAGCAUUC
1 A04	AP2A2	NM_012305	J-012812-05	GAAUUUAGGU CGGAUGUUU	GCCCAUCACU CUCAACAAA	GCACUUGGGU GUGGUAACU	CCGAAUUGCU GGUGAUUAC
1 A05	AP1B1	NM_145730	J-011200-05	UAGACGAGCU UAUCUGCUA	CCACUCAGGA CUCAGAUAA	CUAAGGACUU GGACUACUA	GGAAGGCUG UGCGUGCUAU
1 A06	AP2B1	NM_001282	J-003627-07	GUACAAUGAU CCCAUCUAU	UGAAUUAUGU GGUCCAAGA	CAACAAGUAU GAAAGUAUC	GAUGUUGACU UUGUUCGAA
1 A07	AMPH	NM_139316	J-011569-05	GAACUUCACC CGACGCUUA	UCACAGAGUC GCUGCAUGA	GACAAGCACU GAUUUGGUA	GAGGAUAUUU AGCAGCAAU
1 A08	BIN1	NM_139351	J-008246-05	GACAUCAAGU CACGCAUUG	GAACAGCCGC GUAGGUUUC	CCAGCAACGU GCAGAAGAA	ACAACGACCU GCUGUGGAU
1 A09	ARF1	NM_001658	J-011580-05	UGACAGAGAG CGUGUGAAC	CGGCCGAGAU CACAGACAA	GAACCAGAAG UGAACGCGA	ACGAUCCUCU ACAAGCUUA
1 A10	ARF6	NM_001663	J-004008-05	CGGCAUUACU ACACUGGGA	UCACAUGGUU AACCUCUAA	GAUGAGGGAC GCCAUAAUC	GAGCUGCACC GCAUUAUCA
1 A11	RHOA	NM_001664	J-003860-10	CGACAGCCCU GAUAGUUUA	GACCAAAGAU GGAGUGAGA	GGAAUGAUGA GCACACAAG	GCAGAGAUAU GGCAAACAG
1 B02	ARRB1	NM_020251	J-011971-05	UGGAUAAGGA GAUCUAUUA	AUGGAAAGCU CACCGUCUA	GAACGAGACG CCAGUAGAU	GAACUGCCCU UCACCCUAA
1 B03	ARRB2	NM_199004	J-007292-05	CGAACAAGAU GACCAGGUA	CGGCGUAGAC UUUGAGAUU	UAGAUCACCU GGACAAAGU	GGGCUUGUC CUUCCGCAAA
1 B04	ATM	NM_138292	J-003201-11	GCAAAGCCCU AGUAACAUA	GGUGUGAUCU UCAGUAUAU	GAUGGGAGGC CUAGGAUUU	GAGAGGAGAC AGCUUGUUA
1 B05	ATP6V0A1	NM_005177	J-017618-05	GAACUUACCG AGAGAUAAA	CGGCCGAUGU UUACUUAUA	CCAGCUCCGU AUACUAUUA	GUUCAGUGG UCGAUACAUU
1 B06	CAV1	NM_001753	J-003467-06	CUAAACACCU CAACGAUGA	GCAAAUACGU AGACUCGGA	GCAUCAACUU GCAGAAAGA	GCAGUUGUAC CAUGCAUUA

1	B07	CAV2	NM_198212	J-010958-05	AGAUUGGGAU ACUGUAAUA	GUAAAGACCU GCCUAAUGG	UAUCAUUGCU CCAUUGUGU	GUAGGACGAU GCUUCUCUU
1	B08	CAV3	NM_001234	J-011229-05	UCAAGGUGGU GCUGCGGAA	GCCCAGAUCG UCAAGGAUA	UGCCAUGCAU UAAGAGCUA	GGACAUAGUC AAGGUGGAU
1	B09	CBL	NM_005188	J-003003-09	AAUCAACUCU GAACGGAAA	GACAAUCCCU CACAAUAAA	GGAGACACAU UUCGGAUUA	UAGCCCACCU UAUAUCUUA
1	B10	CBLB	NM_170662	J-003004-09	GAACAUCACA GGACUAUGA	GUACUGGUCC GUUAGCAAA	UAUCAGCAUU UACGACUUA	GGUCGAAUUU UGGGUAUUA
1	B11	CDC42	NM_044472	J-005057-05	CGGAAUAUGU ACCGACUGU	GCAGUCACAG UUAUGAUUG	CUGCAGGGCA AGAGGAUUA	GAUGACCCCU CUACUAUUG
1	C02	CFL1	NM_005507	J-012707-05	CCUCUAUGAU GCAACCUAU	CAUGGAAGCA GGACCAGUA	ACUCUGUGCU UGUCUGUUU	UAAAUGGAAU GUUGUGGAG
1	C03	AP2M1	NM_001025205	J-008170-05	GUUAAGCGGU CCAACAUUU	GCGAGAGGGU AUCAAGUAU	GAACCGAAGC UGAACUACA	AGUUUGAGCU UAUGAGGUA
1	C04	CLTA	NM_001833	J-004002-05	AGACAGUUAU GCAGCUAUU	CCAAUUCUCG GAAGCAAGA	CCAAAGAUGU CUCCCGCAU	AGUAAUGAAU GGUGAAUAC
1	C05	CLTB	NM_001834	J-004003-05	GGAACCAGCG CCAGAGUGA	CAUCUAAGGU CACGGAACA	GGAAACGGCU GCAAGAGCU	GCACAGAGUG GGAGAAGGU
1	C06	CLTC	NM_004859	J-004001-09	GAGAAUGGCU GUACGUAAU	UGAGAAAUGU AAUGCGAAU	CGUAAGAAGG CUCGAGAGU	GCAGAAGAAU CAACGUUAU
1	C07	COPA	NM_004371	J-011835-05	ACUCAGAUCU GGUGUAAUA	GCAAUAUGCU ACACUAUGU	GCGGAGUGGU UCCAAGUUU	GAACAUUCGU GUCAAGAGU
1	C08	DAB2	NM_001343	J-008522-05	GAACCAGCCU UCACCCUUU	CAAAGGAUCU GGGUCAACA	AAACUGAAAU CGGGUGUUG	GAUCUAAACU CUGAAAUCG
1	C09	DIAPH1	NM_005219	J-010347-06	GAAGUGAACU GAUGCGUUU	GAAGUUGUCU GUUGAAGAA	GCGAGCAAGU GGAGAAUAU	GAUAUGAGAG UGCAACUAA
1	C10	DNM1	NM_004408	J-003940-05	GAGAAUCUGU CCUGGUACA	GAAUAUCCAU GGCAUUAGA	CACAGAAUAU GCCGAGUUC	GCAGUUCGCC GUAGACUUU
1	C11	DNM2	NM_001005362	J-004007-05	GGCCCUACGU AGCAAACUA	GAGAUCAGGU GGACACUCU	GAGCGAAUCG UCACCACUU	CCGAAUCAAU CGCAUCUUC
1	D02	EPS15	NM_001981	J-004005-05	AUAAAGAUAU GGACGGAUU	UGAAUUAACU AGUCAGGAA	CUUAAUCAGU CAGAAGUUA	CAAGUGAGGU UCAGGAUCU
1	D03	FYN	NM_153048	J-003140-11	CGGAUUGGCC CGAUUGAUA	GGACUCAUAU GCAAGAUUG	GGAGAGACAG GUUACAUUC	GAAGCCCGCU CCUUGACAA
1	D04	GRB2	NM_203506	J-019220-07	UGAAUGAGCU GGUGGAUUA	AGGCAGAGCU UAAUGGAAA	GAAAGGAGCU UGCCACGGG	CGAAGAAUGU GAUCAGAAC

1	D05	HIP1	NM_005338	J-005001-07	GCAAAUCACA GAUCGAAGA	GAGCCUGUCU GAGAUAGAA	GCAGUGAUCC CUUCAAUUU	GAACAGCGAU AUAGCAAGC
1	D06	LIMK1	NM_016735	J-007730-06	GAGCAUGACC CUCACGAUA	GAAGCGAGUU GCCCGUGUG	GGAGACCGGA UCUUGGAAA	GCCCAGAUGU GAAGAAUUC
1	D07	RAB8A	NM_005370	J-003905-05	CAGGAACGGU UUCGGACGA	GAAUUAAACU GCAGAUAUG	GAACUGGAUU CGCAACAUU	GAACAAGUGU GAUGUGAAU
1	D08	NEDD4	NM_006154	J-007178-06	GGAGGGAACA UACAAAGUA	GAUCACAAUU CCAGAACGA	CCAAUGAUCU AGGGCCUUU	GAACUAGAGC UUCUUAUGU
1	D09	NSF	NM_006178	J-009401-05	GAAAAUCGCC AAUCAAUUA	GGAUAGGAAU CAAGAAGUU	UCUCUUGGCU CGACAGAUU	CAAUAGACCA GAUCUGAUA
1	D10	PAK1	NM_002576	J-003521-09	ACCCAAACAU UGUGAAUUA	GGAGAAAUUA CGAAGCAUA	CAUCAAAUAU CACUAAGUC	UCAAAUAACG GCCUAGACA
1	D11	PIK3C2G	NM_004570	J-006773-05	GAACUUUGCU GUCGUGCUU	GCAAAAGGCU UGAUAGAGA	GAACCCUGCC CUAUGUAUA	ACAACUAGGU CGAUUGAAA
1	E02	PIK3CG	NM_002649	J-005274-07	GCUGAAGCGU GGUUUAAGA	CCCGAAAGCU UUAGAGUUC	GACGUCAGUU CCCAAGUUA	GAAUUGCUCU GGCAUUUUA
1	E03	PI4KA	NM_002650	J-006776-13	GCUAUGUGCG GGAGUAUAU	GAUCGAGCGU CUCAUCACA	GGAACGAAGU GACCCGUCU	GUGGCCAACU GGAGAUCUA
1	E04	RAB1A	NM_004161	J-008283-06	CAGCAUGAAU CCCGAAUAU	GUAGAACAGU CUUUCAUGA	UGAGAAGUCC AAUGUUAAA	GGAAACCAGU GCUAAGAAU
1	E05	RAB2A	NM_002865	J-010533-07	GAAGGAGUCU UUGACAUUA	GCAGGAGCUU UACUAGUUU	GCUUAUUGCU ACAGUUUAC	GUGCUCGAAU GAUAACUAU
1	E06	RAB3A	NM_002866	J-009668-07	GAAGAUGUCC GAGUCGUUG	UCAAGACCAU CUAUCGCAA	GAGGCAAGCG CCAAGGACA	GUUCAAGAUU CUCAUCAUC
1	E07	RAB3B	NM_002867	J-008825-05	GGACACAGAC CCGUCGAUG	CUACUCAGAU CAAGACCUA	UUAAACUGCU UAUCAUUGG	CAAAGGAGAA CAUCAGUGU
1	E08	RAB4A	NM_004578	J-008539-06	GCUCAGGAGU GUGGUUGUU	UACAAUGCGC UUACUAAUU	GAACGAUUCA GGUCCGUGA	GAUAAUAAAU GUUGGUGGU
1	E09	RAB5A	NM_004162	J-004009-05	GCAAGCAAGU CCUAACAUU	UGACACUACA GUAAAGUUU	AGAGUCCGCU GUUGGCAAA	GGAAGAGGAG UAGACCUUA
1	E10	RAB5B	NM_002868	J-004010-06	GGAGCGAUAU CACAGCUUA	GAAAGUCAAG CCUGGUAUU	AAGCUGCAAU CGUGGUUUA	CAACAAACGU AUGGUGGAG
1	E11	RAB6A	NM_002869	J-008975-07	GUGGAUUGAU GAUGUCAGA	CCAAAGAGCU GAAUGUUAU	GAAAGAGGAA GUGAUGUUA	GAGCAAAGCG UUGGAAAGA
1	F02	MAP4K2	NM_004579	J-003587-09	GCGCAAAGGU GGCUACAAU	GGACAGGGAC ACAAUCCUA	CGCCCAAACU GAGAGAUAA	GGAAUGACCG CUUGUGGAU

1	F03	RAB5C	NM_004583	J-004011-07	UCAUUGCACU CGCGGGUAA	GAACAAGAUC UGUCAAUUU	GCUAAGAAGC UUCCCAAGA	GCAAUGAACG UGAACGAAA
1	F04	RAC1	NM_006908	J-003560-14	GUGAUUUCAU AGCGAGUUU	GUAGUUCUCA GAUGCGUAA	GAACUGCUAU UUCCUCUAA	AUGAAAGUGU CACGGGUAA
1	F05	ROCK1	NM_005406	J-003536-06	CUACAAGUGU UGCUAGUUU	UAGCAAUCGU AGAUACUUA	GCCAAUGACU UACUUAGGA	CCAGGAAGGU AUAUGCUAU
1	F06	SEC13	NM_183352	J-012351-05	CAUGUGAGCU GGUCCAUCA	GGUCGUGUGU UCAUUUGGA	GUAAUUAACA CUGUGGAUA	CCAUCUCCCU GCUGACUUA
1	F07	ITSN1	NM_001001 132	J-008365-05	GAUAUCAGAU GUCGAUUGA	GAACGAAAGA UCAUAGAAU	GCACAGAUAU GGGCACUAG	CGACAAGGCC GGAGUCUUC
1	F08	SNX1	NM_148955	J-017518-05	GAAAAGAAGU GAUACGGUU	GGAAAGAGCU AGCGCUGAA	GAAAGGGACU UCGAGAGGA	CAAAGGCCAU CUCCUAAUG
1	F09	SNX2	NM_003100	J-017520-05	CCACAGAAGU UGUAUUAGA	GUGCUGCCAU GUUAGGUAA	UGAAUCGGAU GCAUGGUUU	AAUGAUGGUU GCUAACAAA
1	F10	STAU1	NM_017454	J-011894-05	GCAGGGAGUU UGUGAUGCA	UAAUAAAGAG GAUGAGUUC	CGGAUGCAGU CCACCUAUA	CGAGUAAAGC CUAGAAUCA
1	F11	VAMP1	NM_016830	J-012497-05	UAACAUGACC AGUAACAGA	GGCAGGAGCA UCACAAUUU	GUGGACAUCA UACGUGUGA	CCAUCAUCGU GGUAGUUAU
1	G02	VAMP2	NM_014232	J-012498-05	GCGCAAAUAC UGGUGGAAA	CAUCAUAGUU UACUUCAGC	UCAUGAGGGU GAACGUGGA	GGGAGUGAU UUGCGCCAUC
1	G03	SYT1	NM_005639	J-020044-05	GCAAUUUACU UUCAAGGUA	GGGCACAUCU GAUCCUUAC	GUAAGAGGCU GAAGAAGAA	GAUCGUUUCU CUAAGCAUG
1	G04	TSG101	NM_006292	J-003549-06	CCGUUUAGAU CAAGAAGUA	CUCCAUACCC AUCCGGAUA	CCAAAUACUU CCUACAUGC	CCACAACAAG UUCUCAGUA
1	G05	VAV2	NM_003371	J-005199-05	CUGAAAGUCU GCCACGAUA	UGGCAGCUGU CUUCAUUAA	GCCGCUGGCU CAUCGAUUG	GUGGGAGGG UCGUCUGGUA
1	G06	VCP	NM_007126	J-008727-09	GCAUGUGGGU GCUGACUUA	CAAAUUGGCU GGUGAGUCU	GUAAUCUCUU CGAGGUAUA	CCUGAUUGCU CGAGCUGUA
1	G07	EZR	NM_003379	J-017370-08	GCGCGGAGCU GUCUAGUGA	GCGCAAGGAG GAUGAAGUU	GCUCAAAGAU AAUGCUAUG	GGAAUCAACU AUUUCGAGA
1	G08	WAS	NM_000377	J-028294-09	GCCGAGACCU CUAAACUUA	UGACUGAGUG GCUGAGUUA	GACCUAGCCC AGCUGAUAA	GAAUGGAUUU GACGUGAAC
1	G09	CLTCL1	NM_001835	J-011611-05	CCGAGUGGCU UGUCAAUUU	GCACAUCAUU GAAGUUGGA	GAAUUAAUCC AGCUAACAU	CCAUGAAGAU GUUUGAUAG
1	G10	PICALM	NM_001008 660	J-004004-07	CAACAGGCAU GAUAGGAUA	GUUCAAAGAU GCCAUUAGA	CAUUACAACU CAUCAUUUG	GUAAUGGCCU AUCCUGCUA

1	G11	PIP5K1A	NM_003557	J-004780-09	ACACAGUACU CAGUUGAUA	GCACAACGAG AGCCCUUAA	GUAAGACCCU GCAGCGUGA	GUGGUUCCC UAUUCUAUGU
1	H02	EEA1	NM_003566	J-004012-06	GCAGUCAGCU GGAAAGUCA	GAAGCAACGG UUCAGAAUA	GAACCUUGAA GCUUUAUUA	GUUCAAACAC UAAUGGAUA
1	H03	CAMK1	NM_003656	J-004940-05	AGAUACAGCU CUAGAUAAG	GAAGAUAAGA GGACGCAGA	GAAUGAUGCC AAACUCUUU	UGAAAUACCU GCAUGACCU
1	H04	BECN1	NM_003766	J-010552-05	GAUACCGACU UGUUCCUUA	GGAACUCACA GCUCCAUUA	GAGAGGAGCC AUUUAUUGA	CUAAGGAGCU GCCGUUAUA
1	H05	RAB11A	NM_004663	J-004726-07	GCAACAAUGU GGUUCCUAU	CAAGAGCGAU AUCGAGCUA	GAGAUUUACC GCAUUGUUU	GUGCAGUGC UGUCAGAACA
1	H06	ASAP2	NM_003887	J-011544-05	GAAAUAAGCG GAGCGGAAA	GCAAAGCUCA ACCUGCUAA	CUACGGAUCU UCACACGAU	GAAGGCCUCC AUCGAGAUA
1	H07	SYNJ1	NM_003895	J-019486-07	GAAGCAAUUU CGCAGCAUA	GUUCUGAGCC UAAAUGGUA	UCUCCAAACC CAUUUAUUA	AAACAGAACA GGUUGUGUA
1	H08	SYNJ2	NM_003898	J-012624-05	GGACGUAGCC AUCGACACA	UCACAAGUUU GGACUAUGA	ACCCUAAACU GUUGAAUAA	GAAUUGAGCG CAGGGAAUA
1	H09	AP1M1	NM_032493	J-013196-05	UAUCACGCUU CGAGAAUGA	GCCCAAUGAU GCCGACUCA	GAAGGCAUCA AGUAUCGGA	CGAGAUCCCU UACUUCACU
1	H10	RAB29	NM_003929	J-010556-07	GAGAACGGUU UCACAGGUU	CAGGACAGCU UCAGCAAAC	GCUAGUAGUG UUUGGCUUA	GGACCAGAUU GACCGGUUC
1	H11	WASF1	NM_001024936	J-011557-05	AAACAAGACC UCAGACAUA	CAACUAAGUA GCCUAAGUA	CCAUCAACCC UACCUGUAA	UAGAUUGGUU GGAGUAAGA
2	A02	AP3D1	NM_003938	J-016014-06	CUACAGGGCU CUGGAUAUU	GGACGAGGCA AAAUACAUA	GAAGGACGUU CCCAUGGUA	CAAAGUCGAU GGCAUUCGG
2	A03	HIP1R	NM_003959	J-027079-05	CUGUGGAGAU GUUUGAUUA	UGGCUGACCU CUUCGAUCA	UGAAUGCACU GGAGGGUGA	GCAGGAAUGU UCUCGCACA
2	A04	ATG12	NM_004707	J-010212-06	GAACACCAAG UUUCACUGU	GCAGUAGAGC GAACACGAA	GGGAAGGACU UACGGAUGU	GGGAUGAACC ACAAAGAAA
2	A05	HGS	NM_004712	J-016835-05	GAGGUAAACG UCCGUAACA	GCACGUCUUU CCAGAAUUC	AAAGAACUGU GGCCAGACA	GAACCCACAC GUCGCCUUG
2	A06	VAPB	NM_004738	J-017795-05	UGUUACAGCC UUUCGAUUA	CCACGUAGGU ACUGUGUGA	GCUCUUGGCU CUGGUGGUU	GUAAUUAUUG GGAAGAUUG
2	A07	VAPA	NM_194434	J-021382-05	CCUGAGAGAU GAAGGUUUA	UAGGGAAAUU CAUCUUGUA	GGAUAAACCU GGAUCAACC	GGCAAAACCU GAUGAAUUA
2	A08	RAB11B	NM_004218	J-004727-06	UAACGUAGAG GAAGCAUUC	GAGUACGACU ACCUAUUCA	UCGCCAAGCA CCUGACCUA	CAACUUGUCC UUCAUCGAG

2	A09	CYTH3	NM_004227	J-019268-05	GGGAAUUCAG UUUCUAAUA	GAACGAGCCA UUUAAGAUC	GAGAAGGCCU AAAUAAGAC	AGAGAUCCCU UCUAUGACA
2	A10	ROCK2	NM_004850	J-004610-06	GCAACUGGCU CGUUCAAUU	UAGAAUAUGU GGCCUAGAA	GAAACUAAUA GGACACUAA	CAAACUUGGU AAAGAAUUG
2	A11	MAPK8IP1	NM_005456	J-003595-05	GAAGACUACU GGUACGAGG	AGGACACACU GAAUAAUAA	GAUAUCAUCC AAAGAACAA	GGGAAUAAAU GUAGCCACU
2	B02	RAB3D	NM_004283	J-010822-06	GUUCAAACUG CUACUGAUA	GUACUGUGGG CAUCGAUUU	UGACAUCGCC AAUCAGGAA	GGACGAACGU GUUGUGCCU
2	B03	CLINT1	NM_014666	J-021406-05	GCUCCUAGCU UACCUCAUA	CAGCAGCCAU CACUGAAUA	AUUCAGAGAU CGAGUCUAA	UGGUAAGGAU CAAGGUAUA
2	B04	SNAP91	NM_014841	J-032296-05	GCAUAGACCU GUUUAGUAC	CUACAAUGAU GGUGUUAUU	GCUAAAGAGU AUGCCAAUA	GAGCAAGUUG GUAUUGAUA
2	B05	PDCD6IP	NM_013374	J-004233-09	CAGAUCUGCU UGACAUUUA	UCGAGACGCU CCUGAGAUA	GCGUAUGGCC AGUAUAAUA	GUACCUCAGU CUAUAUUGA
2	B06	AP1M2	NM_005498	J-012056-05	GGUCUUCAUU GAUGUCAUA	CCACUGAUCU GGAUUGAGU	AGAGAAACGU CGUGAUUUG	CCGAGGGUAU CAAGUAUAA
2	B07	ARPC5	NM_005717	J-012080-05	GCAGGCAGCA UUGUCUUGA	GUGUGGAUCU CCUAAUGAA	GAAUAUGACG AGAACAAGU	GCAGUUCAAU CUCUGGACA
2	B08	ARPC4	NM_001024960	J-008571-05	GAACUUCUUU AUCCUUCGA	UAAACCAUCU GGCUGGAUC	GAAGAGUUCC UUAAGAAUU	GAGAUGAAGC UGUCAGUCA
2	B09	ARPC3	NM_005719	J-005284-05	GAUGAGAGCC UAUUUACAA	AAAUGUAUAC GCUGGGAAU	GAAUGAAGCU GAUAGGACC	AUACAGAUAU UGUGGAUGA
2	B10	ARPC1B	NM_005720	J-012082-06	GAGAGUAACC GUAUUGUGA	UAGACUCGCU GCACAAGAA	CGUGUGAUCU CCAUCUGUU	UCGCGACUCU GGCCUCUGA
2	B11	ACTR3	NM_005721	J-012077-06	GCAGUAAAGG AGCGCUAUA	GUGAUUGGCA GCUGUAUUA	GGAAUUGAGU GGUGGUAGA	GCCAAAACCU AUUGAUGUA
2	C02	ACTR2	NM_005722	J-012076-06	GAAAGAGCAU UUAUCGUUU	GAACAUGGAU CUUAGAGUC	AGAAUGGAAU GGACUCUUA	UGGUGUGAC UGUUCGAUAA
2	C03	ARPC2	NM_005731	J-012081-05	CCAUGUAUGU UGAGUCUAA	GCUCUAAGGC CUAUAUUCA	GGACAGAGUC ACAGUAGUC	GUACGGGAG UUUCUUGGUA
2	C04	WASF2	NM_006990	J-012141-05	GGAUUUGGGU CUCCAGGGA	CAAGAGAAGC GGGAUGUUG	GCAAAUGGUU GUAGUAAUU	GGGCAGAGC UUUCUCAGUU
2	C05	EFS	NM_032459	J-012094-05	GAGAUGGUGC AGUGUGUAA	CGUCAGCCUU ACUCAAUUU	GCAAUUCACU ACCCUGCUC	GAUGGAGGAU GACCCAGCA
2	C06	CIB2	NM_006383	J-012230-05	GGGCUUUGCU GACUUCGAG	AAGAGCAGCU AGACAACUA	GCGACAAGGU CAUUGAGGA	GAACCUCACU UUCAACGAC

2	C07	CIB1	NM_006384	J-012261-05	CGGCUUAGUG CGUCUGAGA	GAGCGAAUCU GCAGGGUCU	CCAAAGACAG CCUUAGCUU	UGAACUGCCU CACGGGAGA
2	C08	WASF3	NM_006646	J-012301-06	CAUCGGACGU UACGGAUUA	GCUAACAACU UCUACAUCA	CAGCGAACUU GAAUGUGUA	GGCUGAAGUU CUAUACUGA
2	C09	EPN2	NM_148921	J-004725-05	AGACUACGCU GUUGGAUUU	GAAGAAAGCC GAAGGGACA	CCUUUGAGCU CUUCAGUAA	GAACAAUUAC UCAGAGGCA
2	C10	TNIK	NM_015028	J-004542-10	GAACAUACGG GCAAGUUUA	UAAGCGAGCU CAAAGGUUA	CGACAUACCC AGACUGAUA	GACCGAAGCU CUUGGUUAC
2	C11	ERC1	NM_178038	J-010942-05	GCGGACAAUU GAACGCUUA	UGAAAGAACG GGUCAAAUC	CAAUAUAGCU CUCUUGGAG	GCACAAAUGU UAGAGGAGG
2	D02	MAPK8IP3	NM_033392	J-003596-06	GCAUGGCUGU UGUGUACGA	CAAGAACUAU GCCGAUCAG	GCAGAGCGCA GUCACAUCA	CGAGUGGUC UGAUGUUCAA
2	D03	NEDD4L	NM_015277	J-007187-06	AAGGGAAUAU AUCGACUUA	GAAUAUCGCU GGAGACUCU	GAUCAUAACA CAAAGACUA	GUACAUAUGC GGUCAAAGA
2	D04	AP4E1	NM_007347	J-021474-05	GAGAAUUCAU CUGGAUAUA	UCGAAUACUU UGCACGAUA	CAAGUUAGCC CAACAAGGA	GGUCUAGGAU CAGAAAGUA
2	D05	MAPK8IP2	NM_139124	J-012462-08	AGUUUGAGAU GAUCGAUGA	GGACAGCCCU GACCUCACU	GAAACUGACC GUCCACCUG	ACCAAGAGCA CCUGGCGUA
2	D06	CBLC	NM_012116	J-006962-05	CAUUUGAGCU CUGCAAGAU	GAACAGCAGU GACCAGGAA	GGCCAACACU CCUCAAGAA	GCAACAAGGA UGUGAAGAU
2	D07	ARFIP2	NM_012402	J-012820-05	GCUAGGAGCC GUGAACUUC	CAUUGUGUCU GGUGGCUAU	GCACAAAGCA ACUGUUAUC	GGAGGAAUUU GGCUACAAU
2	D08	DNM3	NM_015569	J-013931-05	GAAAGCUUGU CCUGGUAUA	CGGAAAGGAU UGUUGCUAA	GACCAGGUAU UGCUAUUGA	GGGAUGAGAU GCUUCGAAU
2	D09	RAB11FIP 5	NM_015470	J-004298-05	GUACGUCGGU GGUGGAGAA	CCUGAGCGCC AGUAUGUUU	GCGAUGAGGC CAACCAGAU	GGUACAAGCU GCACUCCAA
2	D10	VPS4A	NM_013245	J-013092-05	CCACAAACAU CCCAUGGGU	CCGAGAAGCU GAAGGAUUA	UCAAAGAGAA CCAGAGUGA	GAAUAACAAU GAUGGGACU
2	D11	GIT1	NM_014030	J-020565-06	GGACGACGCC AUCUAUUCA	CGAGCUGCUU GUAGUGUAU	CCGCACACCC AUUGACUAU	GCUCAGAGAA GAUCCAUUU
2	E02	PACSIN3	NM_016223	J-015343-05	CCAACUACGU GGAGUGUGU	ACAAUCAGCC GGAAAGAGA	GGACAUGGAA CAGGCCUUU	AGACAAAAGC UCAGUAUGA
2	E03	EPN1	NM_013333	J-004724-05	ACUAAUCCCU UCCUCCUAU	GAACGUGCGU GAGAAAGCU	GAUCAAGGUU CGAGAGGCC	GGAAGACGCC GGAGUCAUU
2	E04	PACSIN1	NM_020804	J-007735-05	CGAGAAAGGC CCACAGUAU	CAAGAAGGCC UACCAUUUG	GAACAGCAGC UACAUCCAU	UGACAGAGGC AGACAAGGU

2	E05	ITSN2	NM_147152	J-009841-05	GAUCAAACGU GACAAGUUG	CCAAACAUGU GGGCUAUUA	CCUCAUGGGU CAUCUUAUA	GGUGAAUUAU AGAGCAUUA
2	E06	VPS36	NM_016075	J-004701-07	AAACCGAGCU CGAGGAAUG	CGACUGAUUU GGAGAGAUC	CAAAGAACAU GGCCAGAUU	GGGAAUAGCU AACCCAGUU
2	E07	SH3GLB1	NM_016009	J-017086-05	AGAAUUGGAU GCUCACUUA	UCAACAAGUG GCCUAGUAA	AAACGUCAGC CUUAAAUUU	UUAAGUAGGU GGACUAUGG
2	E08	RAB6B	NM_016577	J-008548-05	GCUGAUAAGA GGCAGAUAA	CAACAGACCU CUAAGUGGA	GAGUUAAGGU UCCAUAAUA	UCAGGAAAGU UGAGUGUAA
2	E09	RAB8B	NM_016530	J-008744-05	GCAAUUGACU AUGGGAUUA	GAACAAUCAC GACAGCGUA	GAUCAAAGAA GACCAGUUU	CGAUAGAACU AGAUGGAAA
2	E10	RAB4B	NM_016154	J-008780-06	GCACUAUCCU CAACAAGAU	AGAAUAAGUU CAAACAGGA	AAUCAUGUCU CCUUCAUCA	UCAGUGACGC GGAGUUAUU
2	E11	EPN3	NM_017957	J-021006-05	GUACAAGGCU CUAACAUUG	GAACCGUCCU GUCCCGAAG	CUAGUUCGCU CAUGUCCGA	GGACUUGGC UGACAUCUUC
2	F02	SAR1A	NM_020150	J-016756-05	GAGCAAGCAC GUCGCGUUU	UAUAUUGACU GAUGUUUGG	GAGGAUGUCU UUAUUCUAA	GCAUGCAUUU CGUUUAUUA
2	F03	SH3GLB2	NM_020145	J-015810-05	GCAAAGCUCG GGUGCUCUA	GACUAGACCU CGUAAUUAC	GCUCUGGAAU GAUGAAGUG	CCACGACGGU GCCUGACUU
2	F04	EPS15L1	NM_021235	J-004006-05	GAAGUUACCU UGAGCAAUC	CAAUAGUGCU GAAGGCUUU	GUAAAGGGUU CUUGGACAA	GCAACAACAC GCAAGAGUU
2	F05	GORASP1	NM_031899	J-013510-05	GAUCUCUACC ACAGAAUAA	GAGGACUUCU UUACGCUCA	GAACUGACCA CCACAGCUG	CUGGAGGUG UUCAAUAUGA
2	F06	MAP1LC3A	NM_181509	J-013579-05	GGACGGCUUC CUCUAUAUG	CGGUGAUCAU CGAGCGCUA	UCGCGGACAU CUACGAGCA	UGAGCGAGUU GGUCAAGAU
2	F07	RAB3C	NM_138453	J-008520-05	UGAGCGAGGU CAACAUUUA	GGAUCGAUUU CAAAGUAAA	GUACAAGAUU GGUCAACUC	GCCAUGGGC UUUAUUUUAA
2	F08	IP6K3	NM_054111	J-006739-05	GGAAUGAGCA CACCACCUA	ACAUGAGCGU GAUGAAGUA	UCUAUCAGUU CCUACAUAA	GUUCAUACCG CUUCUAUUC
2	F09	CIB3	NM_054113	J-012901-05	CCCGCGACCU CAAGGCUUA	UCAUGAGGCU CUUCUAUCG	GUGAGAAGGU GCUGGAUGA	CCAGAGGAUU GCCCAGGUA
2	F10	SYT2	NM_177402	J-018809-05	GUAAAGGUGC CUAUGAACA	GAUCGCCAUU GCUGUGGUU	GAACGAAGCC AUAGGCAAG	AGACCAAAGU CCAUCGGAA
2	F11	RAB7B	NM_177403	J-018225-05	GUAGGGCUCU GUCGAGGUA	GAAACUCAUU AUCGUCGGA	UCAAUGUGGU GCAAGCGUU	GGAAGUAGCU CAAGGCUGG

2.6 Western Blot

The cells were scraped in 1ml Hypotonic Buffer (10mM HEPES, 15mM KCl, 10mM MgCl2, 1mM EDTA) +PIC. The cells were the homogenized through a 25 gauge needle 15 times and centrifuged @500 rcf for 5 min to pellet the nuclei, the supernatant was then transferred to a new 1.5 ml tube and enough 10%SDS was added to achieve 2% final concentration. BCA was used to calculate protein concentration. 12% SDS-PAGE gels were prepared with a 15-well comb. The samples were thawed and heated to 65C for 10min. The samples were then loaded in the gel. The gel was run until the dye front run off at the bottom of the gel, then the gel was transferred to a low fluorescence PVDF membrane by wet transfer in Towbin buffer. After transfer the membrane was cut in four and blocked 3 with 5%BSA and one with 5% Milk in TBS-T. After 1 hr blocking at RT the membranes were probed overnight with primary antibody. The blots were washed 3X for 10 min with their respective blocking buffer then secondary HRP antibody was added to the respective blocking buffer to the respective primary (rabbit for 1-2 and mouse for 3-4) and incubated in rocker at RT for 1hr. After the blots were washed 3X form 10min with TBS-T. To image BioRad ECL solution was added and left for 1 min and then imaged with BioRAD ChemiDoc MP, same software was used for quantification.

2.7 Immunofluorescence.

For immune labeling of the different intracellular compartments, I used Rab3, Rab4, Rab5, Rab7, Rab9, and Rab11 antibodies from CST and antibody against the lysosomal marker LAMP1 from Santa Cruz Biotechnology. Alexa-labeled secondary

antibodies were purchased from Invitrogen. Before immune labeling, cells were fixed using a mild fixation method; briefly, cells were kept in media at 37°C, and ice-cold 8% PFA was added dropwise. To preserve peripheral endosomes, 0.025% saponin was used as a mild cell permeabilization agent.

2.8 RT-PCR and qPCR

Two sets of primers specific to each isoform, isolated RNA from HEK-293 cells and other human cell lines, and used purchased total human RNA as a positive control. The RNA was used to create cDNA, which was used as the template for polymerase chain reaction with the specific primers.

2.9 Acyl–Biotinyl Exchange to Analyze Palmitoylation

Palmitoylation analysis was done using acyl–biotinyl exchange (ABE) as previously described (Wan, Roth, Bailey, & Davis, 2007). One 10cm plate with HEK-293 cells at 80-90% confluency was lysed in 2% SDS-containing buffer, and free cysteines were blocked by 10 mM NEM. Then, palmitoylated cysteines were liberated by 0.4 M hydroxylamine and labeled with biotin-HPDP (Pierce). Biotinylated proteins were pulled down using streptavidin-magnetic beads (Dynabeads from Thermo Scientific) and eluted with 1% 2-mercaptoethanol. Three chloroform/methanol precipitations were performed between each step to remove chemicals. After elution, a western blot of the eluate (palmitoylated fraction) and input (total protein) was performed for the different Rab proteins (endogenous). For quantification, densitometry analysis was performed in BioRAD ChemiDoc MP, and the palmitoylated signal was

divided by the input; then this ratio was normalized to the same ratio for endogenous calnexin a known palmitoylated protein.

2.10 Retention using selective hooks (RUSH)

TMD probes constructs linked to streptavidin binding peptide (SBP), and the binding of the peptide to streptavidin sequesters the probe in the cellular compartment. This hook is then released by the addition of biotin to the media. I have used a hook at the endoplasmic reticulum to synchronize the trafficking of raft-TMD as well as non-raft-TMD. Plasmid with a KDEL-tagged avidin that co-expresses the SBP-TMD-fluorescent protein were transfected in HEK after 16 hours the cells were imaged under a fluorescent microscope. Images were taken before the addition of biotin and every hours after 40uM(final concentration) biotin were added to the media until a steady distribution of the probe was reached (Boncompain et al., 2012).

Chapter 3

Raft affinity is a determinant of PM recycling

3.1 Introduction

The cellular localization of bitopic proteins is correlated to their TMD length(Munro, 1995; Sharpe, Stevens, & Munro, 2010), with longer TMDs targeting proteins to the PM and shorter TMDs found in the endoplasmic reticulum (ER), Golgi apparatus, and endocytic organelles. These findings suggest cargo sorting in the secretory and endocytic pathways, with proteins containing longer TMDs, together with sphingolipids and cholesterol, being specifically trafficked to the PM. One possibility for sorting of specific lipid classes along with proteins containing longer TMDs is lateral segregation and coalescence of ordered domains. Because ordered phases in lipid model systems are 0.6–1.5 nm thicker than disordered domains (Garcia-Saez, Chiantia, & Schwille, 2007), raft-associated TM proteins would be predicted to have longer TMDs. Proteins using this “raft pathway” would not require cytosolic sorting signals but rather would be recruited to vesicle budding platforms by their raft affinity, i.e., their preferential interaction with specific lipids or other raft embedded proteins.

Our previous work has explored the structural determinants of transmembrane protein partitioning to ordered membrane microdomains known as lipid rafts. We show that indeed TMD domain length is a determinant of raft partitioning (Diaz-Rohrer, Levental, Simons, & Levental, 2014; Lorent et al., 2017). Using GPMVs, which are intact, isolated PM blebs that phase separate into coexisting ordered and disordered phases that sort lipids and proteins, we were able to measure raft affinity in the protein’s native environment. GPMV corroborate previous observations that saturated lipids, glycolipids, sterols, GPI-anchored proteins, palmitoylated proteins, and

transmembrane proteins with specific structural features are recruited to ordered domains, whereas unsaturated lipids, transferrin receptor, and most other transmembrane proteins are largely excluded (Levental, Lingwood, Grzybek, Coskun, & Simons, 2010).

3.2 Results

In order to study the effect of raft affinity in protein localization I created probes composed of a TMD linked by a small amino acid linker to a fluorescent protein (schematized in Fig 2A). Each probe was expressed in HEK-293 cells to test their subcellular localization. Simultaneously, GPMV were obtained from HEK cells expressing the various TMD probes to test the raft affinity of each construct. An example for the quantification of the affinity of proteins for the raft phase is shown in Fig 2B. RFP-labeled TMDs were expressed in HEK cells and GPMVs were isolated after counter-staining the cells with FAST-DiO (DiO), an unsaturated fluorophore that labels the non-raft phase. Raft affinity is calculated as the ratio between RFP intensity in the raft versus non-raft phase.

3.2.1 Raft affinity is a determinant of PM localization

Consistent with our previous reports, certain natural (LAT and PAG shown) and synthetic TMDs (allA8L) can partition efficiently to rafts phases, whereas other TMDs are excluded (allLeu, delta6, LDLR) (Fig 2C). Sequences of the TMDs used in our studies are listed in Table 4. Strikingly, the subcellular localization of these various TMDs correlated perfectly with their raft affinity. Raft-associated TMDs were localized

at the PM, whereas all TMDs with minimal raft affinity were accumulated in distinct intracellular puncta (Fig 2D-E).

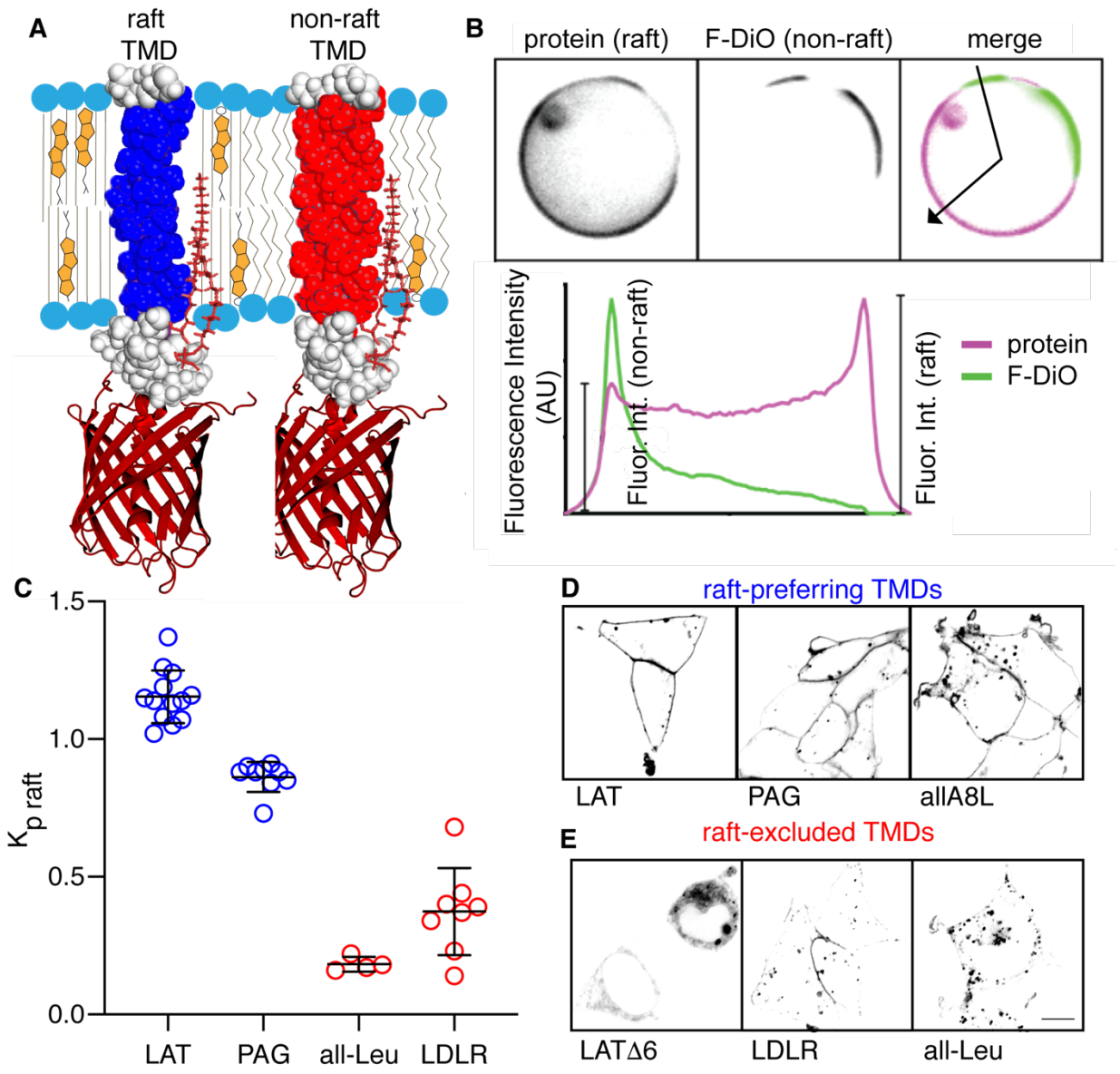


Figure 2. Raft association is sufficient for PM localization. (A) Schematic of TMD constructs composed of a TMD attached to a fluorescent protein. (B) Representation of Kp calculation. Normalized line scans of the protein intensity along the black line in the merged images the two peaks corresponding to raft and nonraft intensity, respectively. Background subtracted ratios of these two intensities yield raft partition coefficients, Kp,raft. (C) Kp measurements for various TMD constructs demonstrate that the TMD of

previously raft-associated proteins are sufficient for raft partitioning. (D) TMDs with high raft affinity localize to the PM while (E) TMD constructs with low raft affinity are predominantly localized to intracellular membranes.

Table 4. TMD amino acid sequences

	Extracellular																											Cytoplasmic
LAT	M	E	E	A	I	L	V	P	C	V	L	G	L	L	L	L	P	I	L	A	M	L	M	A	L	C	V	H	C
PAG	Q	I	T	L	W	G	S	L	A	A	V	A	I	F	F	V	I	T	F	L	I	F	L	C	S	S	C		
allA8L	M	E	E	L	A	A	L	A	A	L	A	A	L	A	A	L	A	A	L	A	A	L	A	A	L	C	V	H	C
LATd6exo	M	E	E	V	L	G	L	L	L	L	P	I	L	A	M	L	M	A	L	C	V	H	C	H					
LDLR	M	E	E	A	L	S	I	V	L	P	I	V	L	L	V	F	L	C	L	G	V	F	L	L	W	C	V	H	C
All-Leu	M	E	E	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	C	V	H	C

These results emphasize the remarkable fact that constructs composed solely of TMDs fully recapitulate the PM localization of their parent proteins (LAT and PAG), i.e. these TMDs are sufficient for steady-state localization, containing all essential sorting signals for proper protein trafficking. The sorting signal in these TMDs appear to be their affinity for raft domains, as constructs with abrogated raft affinity (e.g. via truncation, as for delta6), or TMDs with no intrinsic raft affinity (e.g. all-Leu), fail to localize to the PM.

3.2.2 Abrogation of raft affinity results in mis-sorting to lysosomes

To further detail the localization of the non-raft TMD probes that fail to reach the PM, I created HEK cell lines that constitutively expressed either the TMD from LAT (enriched in the raft phase) or a synthetic TMD composed solely of Leu resides (all-Leu) which is almost completely excluded from raft domains. This behavior is shown in Fig 3A.

I used this cell lines to identify the intracellular puncta that the non-raft TMD was accumulating in. These puncta colocalized with markers of late endosomes and

lysosomes (LAMP1) (Fig 3B). The absence of either construct in the Golgi compartment suggested to us that the distinct localization was not related to their secretory trafficking.

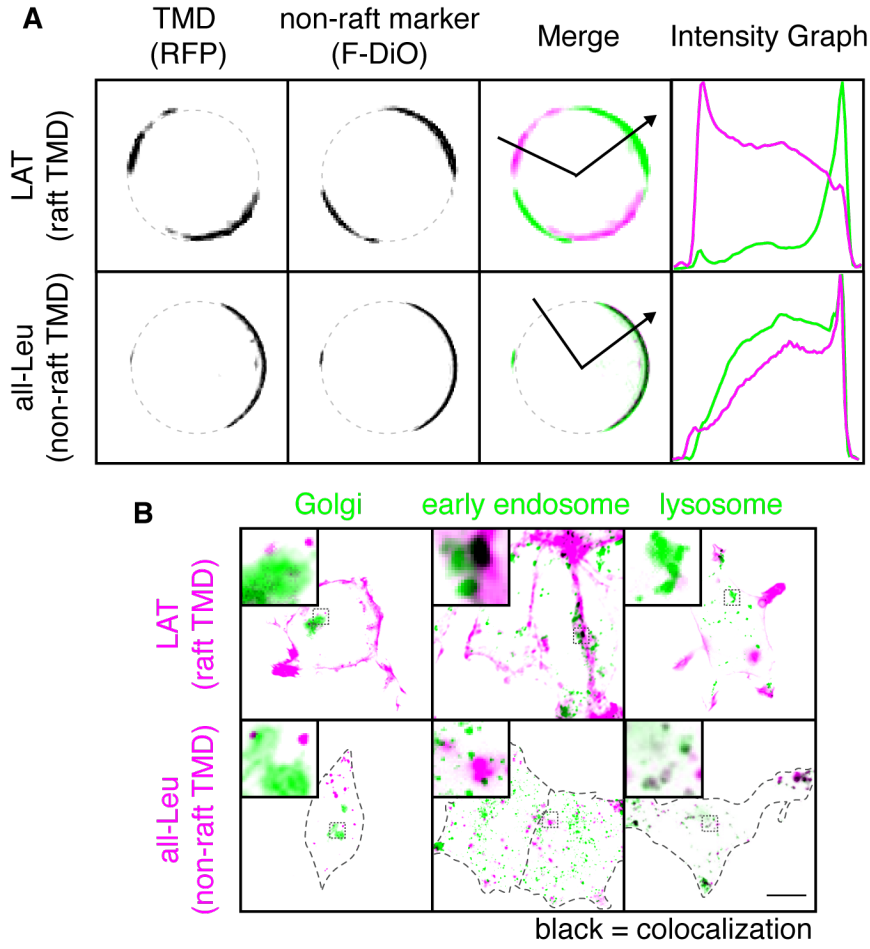


Figure 3. Non-raft TMD is trafficked to lysosomes for degradation. (A) Representative images of GPMVs isolated from cells expressing LAT TMD or AllL TMD. (First column) TMD (magenta). (Second column) Unsaturated lipid marker FAST-DiO (F-DiO; green) to visualize the nonraft phase. (Third column) merge of first two columns. (Fourth column) Normalized line scans of the protein intensity along the dashed black lines in the merged images the two peaks corresponding to raft and nonraft intensity, respectively. (B) Steady state cellular localization, the raft probe

localizes to the PM and early endosomes and the non-raft to the late endosome and lysosome.

3.2.3 Raft TMD and non-raft TMD are sorted in the endosomal system

To further investigate the mechanism that leads to lysosomal localization of the non-raft mutants, I blocked endocytic maturation with the inhibitors bafilomycin A1 and brefeldin A. These inhibitors have different targets but similar effects, affecting the progression from early to late endosome, effectively causing a traffic jam in the endocytic system. Treatment with either inhibitor resulted in relocalization of all-Leu from the lysosomes to the PM after treatment for two hours (Fig. 4 A-B). Thus, blocking proper endosomal sorting led to colocalization of raft and non-raft TMDs, suggesting that sorting of raft proteins away from other membrane components occurs in the endocytic system. Both raft and non-raft TMDs could be observed localized to Rab5-positive vesicles (Fig 3B), suggesting that sorting occurs at the early endosome (EE) or a later compartment in the endocytic pathway. I used a different inhibitor (Wortmannin) to block sorting from the early endosome. Treatment with this inhibitor caused accumulation of both raft TMD and non-raft TMD in enlarged intracellular vesicles after treatment for one hour, presumably early endosomes (Fig 4C). The intracellular accumulation and number of vesicles increased for the duration of the treatment with the inhibitor. These observations suggest that both probes get endocytosed and that after the early endosomes their trafficking routes diverge, and that partition to lipid rafts targets the raft TMD for recycling to the PM. If the TMD fails to partition to lipid rafts, and in the absence of other sorting signals, the protein is targeted to lysosomes.

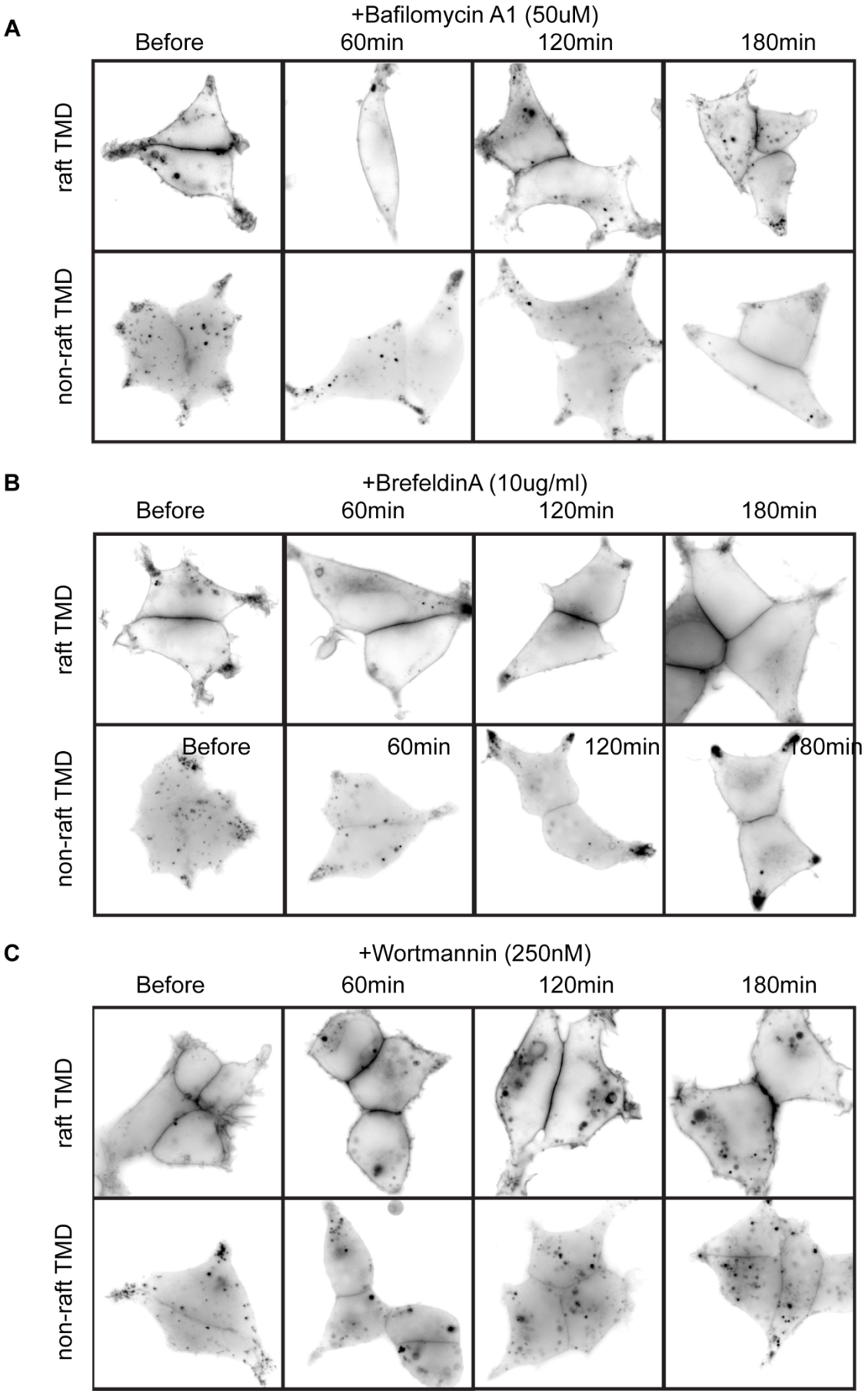

A
+Bafilomycin A1 (50uM)
Before
60min
120min
180min
raft TMD
non-raft TMD
B
+BrefeldinA (10ug/ml)
Before
60min
120min
180min
raft TMD
non-raft TMD
Before
60min
120min
180min
C
+Wortmannin (250nM)
Before
60min
120min
180min
raft TMD
non-raft TMD

Figure 4. non-raft TMD fails to recycle after endocytosis. (A and B) Perturbation of endosomal traffic with Bafilomycin A1 and Brefeldin A caused redistribution of non-raft TMD to the PM after 2 hours. (C) Inhibition of sorting form the EE with Wortmannin resulted in enlarged EE that accumulated both raft TMD and non-raft TMD after one hour that increased over the 3-hour treatment.

3.2.4 Raft TMD and non-raft TMD reach the PM

To further demonstrate that the non-raft TMD can reach the PM I used the retention using selective hooks (RUSH) system to synchronize protein trafficking from the ER (Boncompain et al., 2012). This method uses a streptavidin hook linked to a retention peptide sequence (KDEL) to keep the "hook" at the ER. The TMD probes were then tagged with a streptavidin binding peptide on the ER lumen/extracellular side of the TMD. Thus, binding of the peptide to streptavidin sequesters the TMD probes in the ER. This interaction between the hook and TMD can then be released by the addition of biotin to the media. Using this setup, I captured both raft TMD and non-raft TMDs at the ER (Fig 5A-B, -biotin). 30 min after the addition of biotin, both probes concentrate in the perinuclear region, indicating trafficking form the ER to the Golgi (Fig. 5 A-B second panel). Fluorescent signal can be seen at the PM for both probes after 3 hours and more noticeable after 6 hours. After 21 hours the raft-TMD is mostly localized to the PM while the non-raft TMD is in intracellular vesicles (Fig 5 A-B last panel), replicating my observations from steady state distribution, but also confirming that the non-raft TMDs reach lysosomes after arriving at the PM.

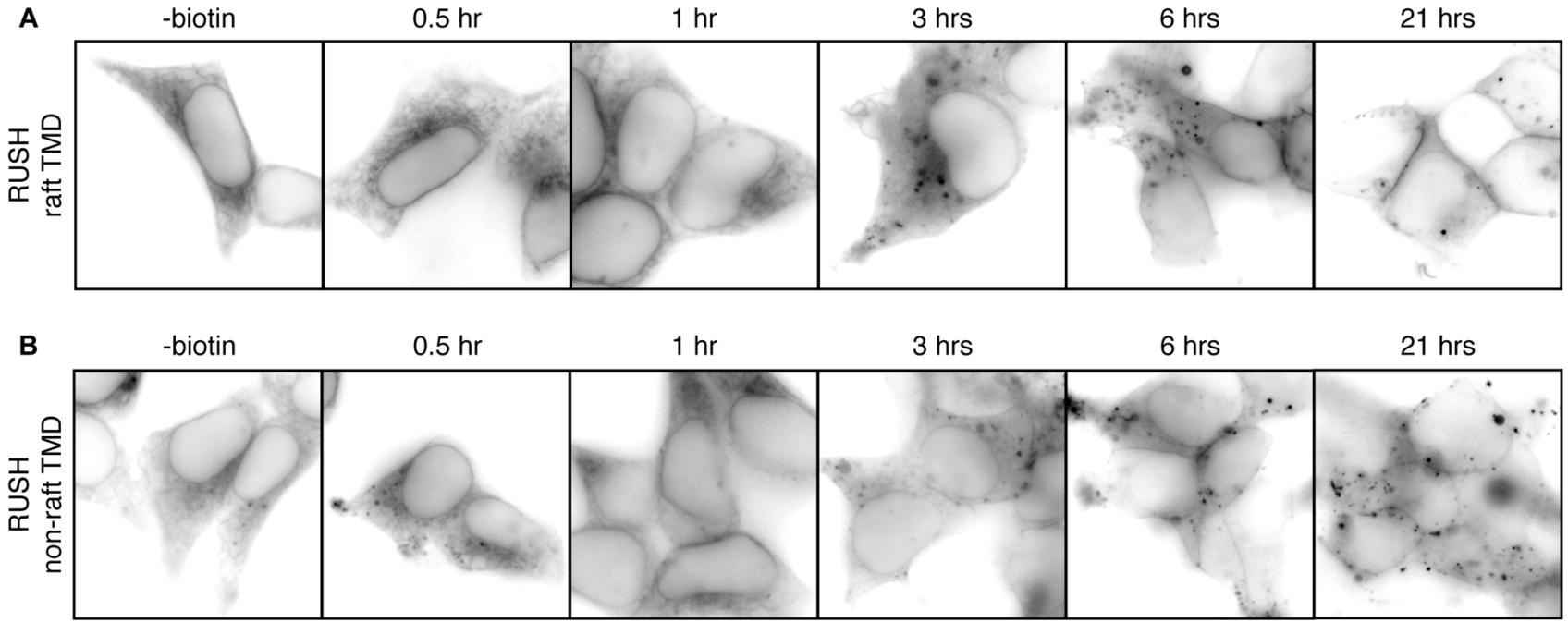


Figure 5. Raft TMD and non-raft TMD traffic to the PM. (A-B) RUSH was used to capture raft TMD (A) and non-raft TMD (B) at the ER after protein translation, the addition of biotin releases the hook and results in synchronized trafficking. At 3 hours both raft TMD and non-raft TMD have reached the PM and after 21 hours their localization mimics that of the steady state distribution.

3.2.5 Raft mediated trafficking can also sort lipids components

All known recycling routes require a sorting signal. These signals depend on a peptide sequence recognized by an adaptor protein, which can then sort a protein to a particular recycling compartment/pathway. Most of the work in understanding how proteins are sorted into diverse subcellular compartments has been done using a specific protein. Even though much has been learned using this approach, it has limitations; among all single-pass transmembrane proteins that are annotated to localize at the PM, less than 20% have a known recycling motif (Fig. 6A). For the remainder, it remains unclear how these proteins are maintained at the PM despite rapid and constant endocytic flux. The TMD constructs examined in this work have no residues available for protein-protein interactions. All constructs contain the same cytosolic structure, consisting of a short linker to a fluorescent protein. The only differences among these constructs are in their TMDs. Thus, these TMDs are their trafficking determinants, by mediating their partitioning to lateral subdomains within a membrane.

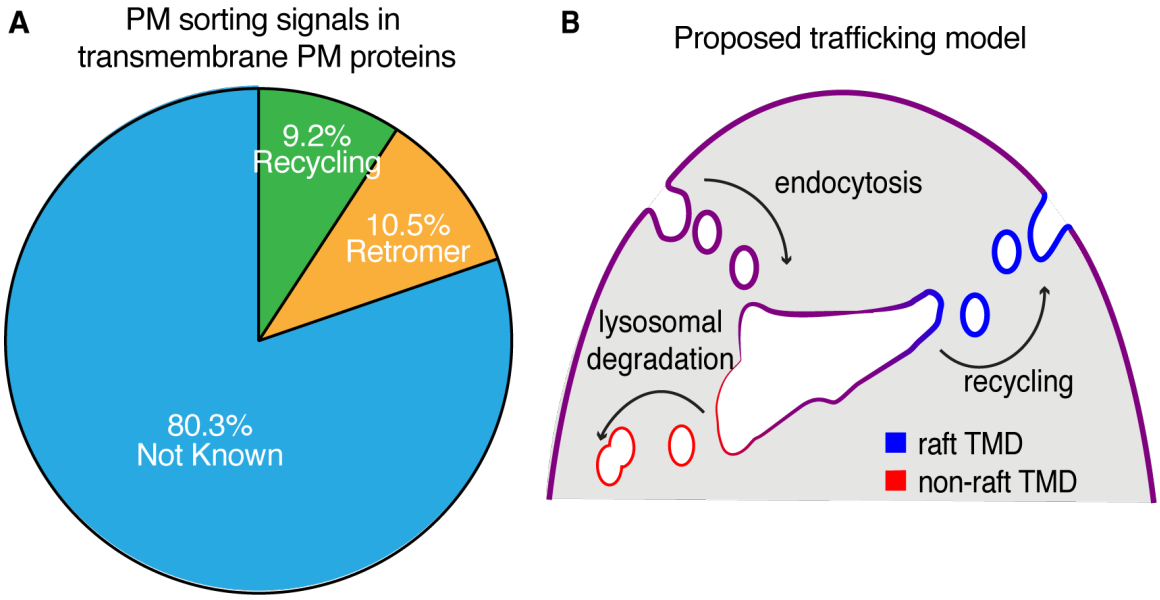


Figure 7. Majority of proteins lack PM sorting signals. (A) The percentage of PM transmembrane proteins that contain various sorting signals. For >80% of such proteins, no PM sorting seqeunce is known. (B) Scheme of raft-dependent recycling.

3.3 Summary

The idea that lipid rafts can serve as a sorting platform between intracellular compartments is not novel, and the evidence that not one but various TMDs from different proteins can localize to the PM based only on their ability to associate with lipid rafts clearly demonstrates that there is a raft-sorting recycling pathway. The PM itself is highly enriched in lipid raft components (cholesterol and sphingomyelin), and as seen in our previous work, the single-pass transmembrane proteins located at the PM are, as a collection, more likely to be in a lipid raft than the single-pass transmembrane proteins located at other intracellular compartments(Lorent et al., 2017). For some of those 80% of proteins without a known recycling motif, lipid raft affinity may explain this maintenance at the PM.

Chapter 4

Raft components are sorted at the late endosomes

4.1 Introduction

The early endosome has been known as the sorting compartment that is shared for both the degradation and recycling pathways. This hypothesis has been supported by lipid composition analysis, which has shown raft components present to varying degrees on the membranes of different compartments. Cholesterol and sphingomyelin are present at the EE, are enriched at recycling endosomes, and are depleted from late endosomes (Gagescu00, Kobayashi99). Further studies have shown that segregation of receptor and ligand occurs in early endosomes in less than 3 min (Yamashiro87). Based on the central role of the early endosomes in sorting of PM components, I hypothesized that the early endosome was the location of raft-based TMD sorting. The results I obtained from testing this hypothesis are described in this chapter.

4.2 Results

4.2.1 Raft and non-raft TMDs traffic through late endosomes

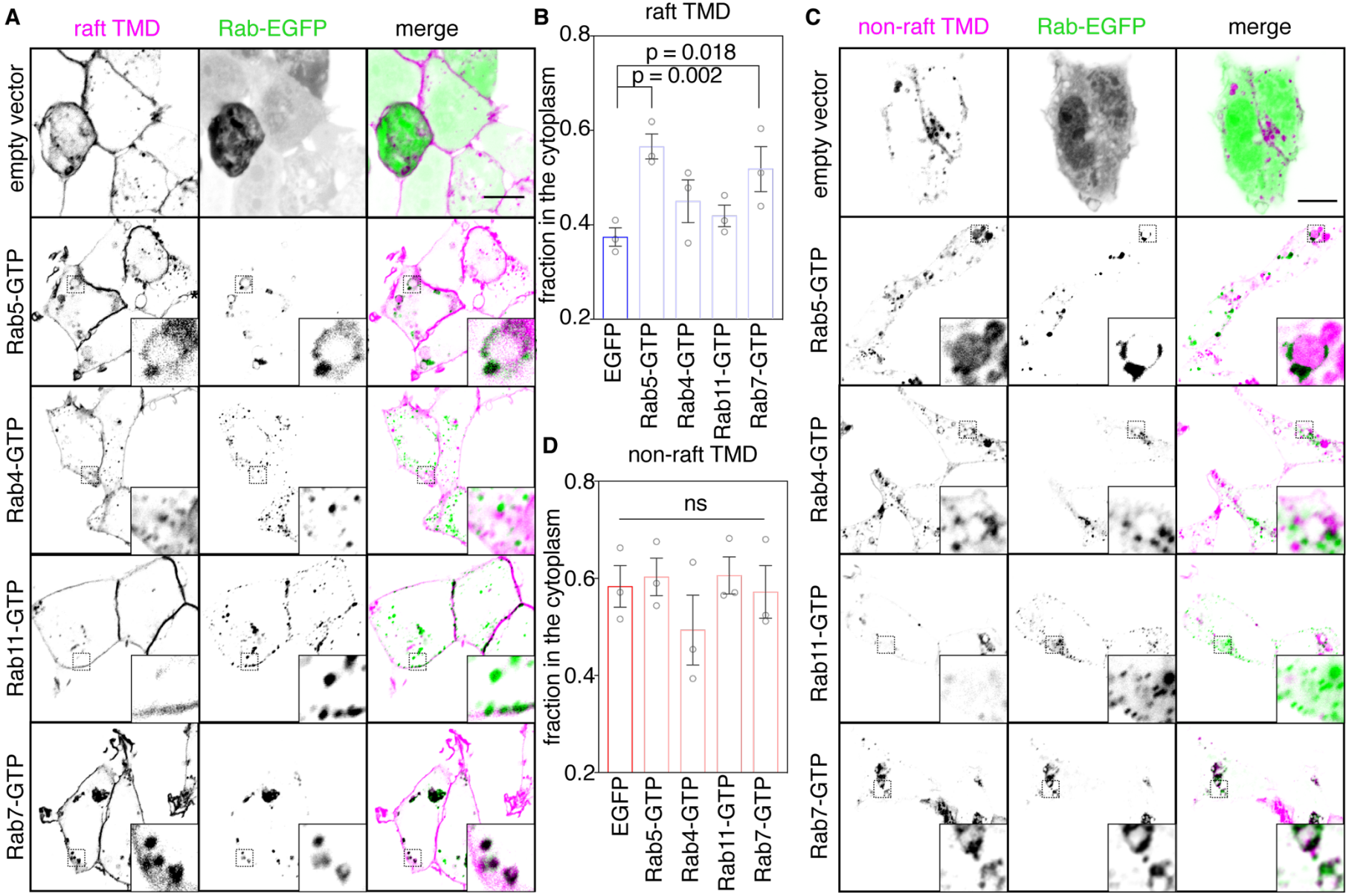
To elucidate the endocytic compartment at which raft-based sorting occurs, I relied on Rab-GTPases, which are known as the molecular labels of intracellular compartments. I overexpressed wild-type and two dominant negative mutants (GDP and GTP locked) of the different endocytic Rab-GTPases in cellular clones expressing LAT_{TMD} and all-Leu, hereafter called raft TMD and non-raft TMD, respectively. For this experiment we used Rab5 (an effector of early endosomes), Rab7 (an effector of late endosomes), and Rab4 and Rab11 (effectors of two well-known recycling pathways). In

cells expressing GTP-locked Rab5, both raft TMD and non-raft TMD probes accumulated in Rab5-positive compartments (Fig.7A and C, top), which we expected because most endocytic traffic passes through this compartment and also because most PM sorting occurs in this compartment.

In contrast and to our surprise, expression of GTP-locked Rab4 and Rab11 had no effect on raft TMD localization (Fig. 7C, middle), suggesting that raft-mediated recycling occurs via a distinct pathway to most known PM recycling. Overexpression of GTP-locked Rab11 had no effect on non-raft TMD localization, but overexpression of GTP-locked Rab4 caused a small increase in the fraction of non-raft TMD at the PM (Fig. 7A middle).

Most surprisingly, perturbation of Rab7 had a similar effect to Rab5. Specifically, overexpression of GTP-locked Rab7 led to an accumulation of both raft TMD and non-raft TMD probes in Rab7-positive compartments (Fig. 7A and C, bottom). All these results were quantified in Figure 7 panel B and D. The observation that both raft TMD and non-raft TMD accumulate in the late endosome after Rab7 overexpression suggests that raft-mediated TMD sorting occurs at late endosomes, unlike the most known PM proteins.

Figure 7 - Raft and non-raft endocytic cargo reach the late endosome compartment. (A) Cells expressing raft TMD were transfected with EGFP-tagged Rab mutants locked in the GTP bound (or empty EGFP vector). Left column (pink) shows the localization of raft TMD. Center column shows localization of Rab in transfected cells. Rab5- and Rab7-GTP overexpression leads to accumulation of raft TMD in endosomes marked by those proteins. (B) Quantification of the fraction of raft TMD fluorescence in the cytoplasm. Each dot represents the average for each separate experiment with 50-100 cells each. The p value was calculated by t-test comparing transfected cells to empty vector. (C) Cells expressing non-raft TMD were transfected with EGFP-tagged Rab mutants locked in the GTP bound (or empty EGFP vector). Left column (pink) shows the localization of non-raft TMD. Center column shows localization of Rab in transfected cells. Rab11-GTP overexpression leads to increase of non-raft TMD in the PM. (D) Quantification of the fraction of non-raft TMD fluorescence in the cytoplasm. Each dot represents the average for each separate experiment with 50-100 cells each. The p value was calculated by t-test comparing transfected cells to empty vector.


A
raft TMD
Rab-EGFP
merge
empty vector
Rab5-GTP
Rab4-GTP
Rab11-GTP
Rab7-GTP
B
raft TMD
p = 0.018
p = 0.002
fraction in the cytoplasm
0.8
0.6
0.4
0.2
EGFP
Rab5-GTP
Rab4-GTP
Rab11-GTP
Rab7-GTP
C
non-raft TMD
Rab-EGFP
merge
empty vector
Rab5-GTP
Rab4-GTP
Rab11-GTP
Rab7-GTP
D
non-raft TMD
ns
fraction in the cytoplasm
0.8
0.6
0.4
0.2
EGFP
Rab5-GTP
Rab4-GTP
Rab11-GTP
Rab7-GTP

4.2.2 Raft TMD and non-raft TMD are sorted at the late endosome

Using the same cell clones, we confirmed the presence of raft TMD and non-raft TMD in native Rab7-positive vesicles by immunostaining (Fig. 8A). The participation of Rab7 in raft-mediated trafficking was further confirmed using siRNA knockdown. Knockdown of Rab7 caused intracellular accumulation of both raft TMD and non-raft TMD (Fig. 8B). These results suggest that the sorting of raft TMD away from non-raft TMD occurs at the late endosome and requires a functional Rab7.

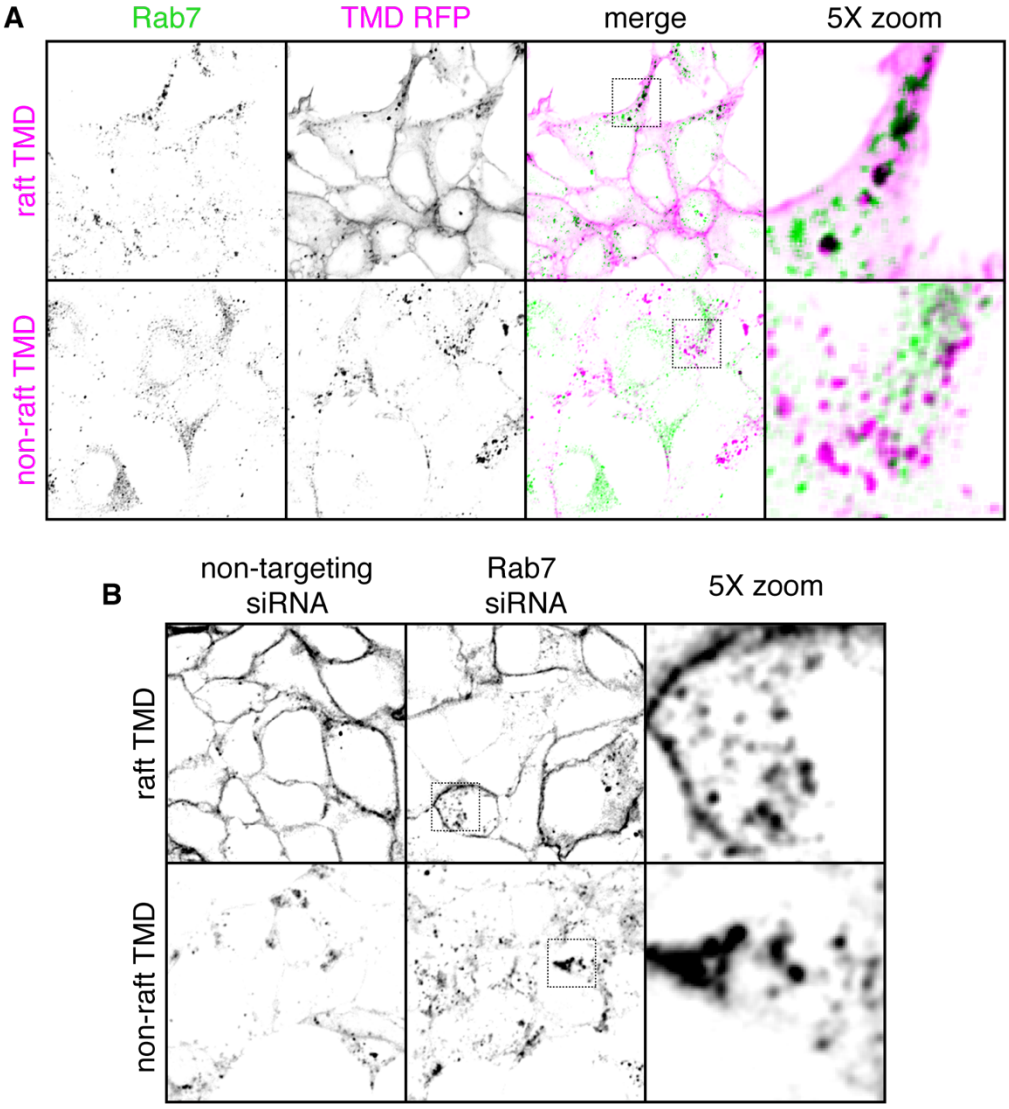


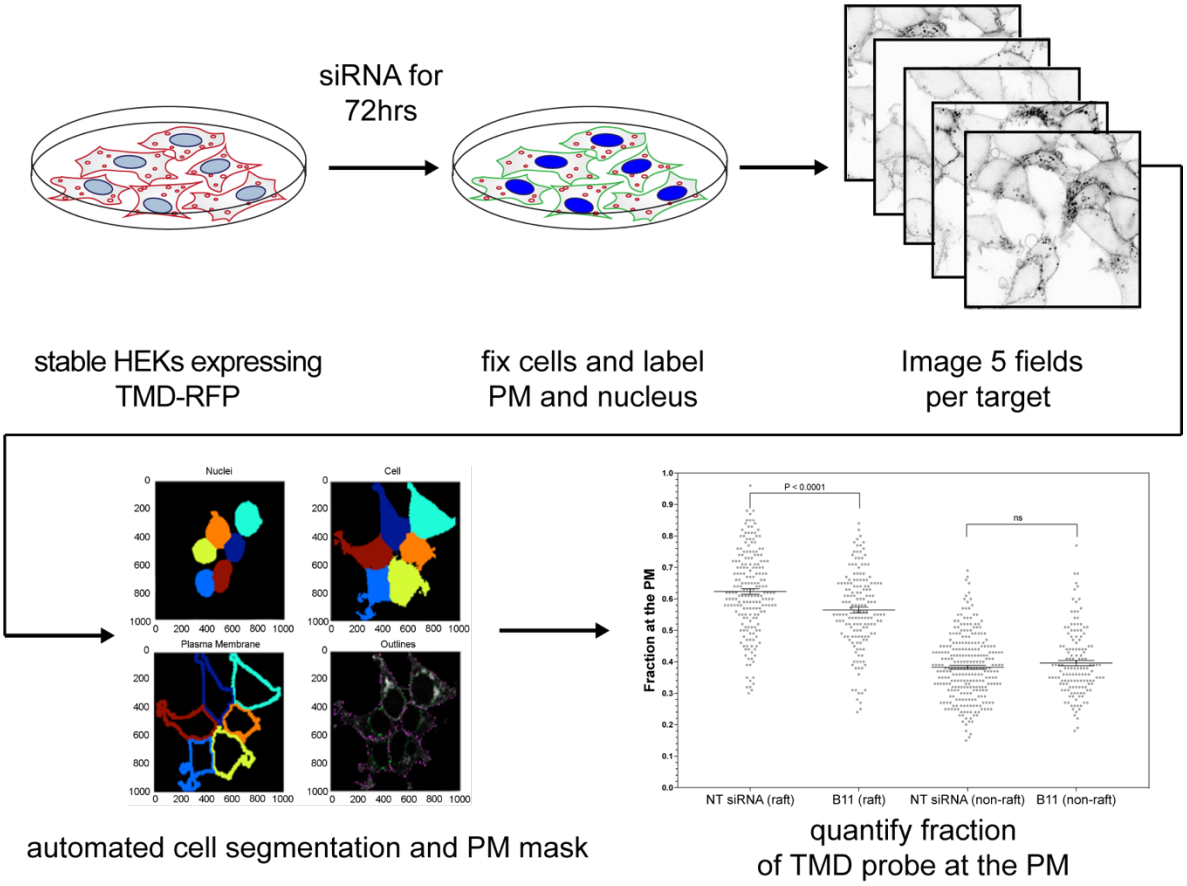
Figure 8 - Raft and non-raft endocytic cargo sort at the late endosome. (A) Cells expressing either raft TMD or non-raft TMD were immunostained for Rab7, revealing that at steady state raft TMD can be found in late endosome. (B) Rab7was knockdown using siRNA to test its involvement in TMD trafficking. Rab7 knockdown led to intracellular accumulation of raft TMD.

4.2.3 Determining the lipid raft mediated recycling machinery

Sorting is the first step to transfer components form one organelle to another, but other steps are required, including fission, targeting and fusion. All these processes involve the recruitment of specific machinery. Our observations suggest that lipid rafts serve as platforms for protein sorting in late endosomes, implying that other raft-resident proteins are necessary to recruit the trafficking machinery required for vesicle formation and targeting. In order to identify this machinery, we will use a candidate-free approach to test a large number of potential candidates.

Using the clonal cell lines expressing raft TMD and non-raft TMD, we developed a high-throughput siRNA mediated knock-down screen to dissect the molecular machinery for raft-mediated sorting. Using siRNA pools for 156 proteins previously implicated in membrane trafficking, we knocked down individual target proteins and assessed their role in raft-mediated recycling by changes in the steady-state localization of raft TMD. Specificity for the raft pathway was evaluated via lack of effect on the localization of non-raft TMD. The workflow of the experiment can be seen in Fig. 9A.

Figure 9. High throughput screen. Experimental flow through for identification of effectors of raft dependent trafficking.



We identified and validated a number of hits, as well as novel players that appear to define a distinct class of trafficking mediators specific to raft-associated proteins (Fig. 10). The list of positive hits is shown as Table 5. Proteins that play are role in early endocytic traffic (Rab5 and EEA1), affected trafficking of both raft and non-raft TMD as expected which served as a positive control. This also supported or findings in from figure 7 that both raft and non-raft TMD traffic through the early endosome.

We focused our validation on three GTPases that were among the most specific and robust hits, namely Arf6, Rab3A, and Rab3B. These proteins were chosen because

(a) GTPases play central roles in defining and mediating vesicle traffic, (b) Arf6 has been previously implicated in PM recycling, specifically in trafficking of cholesterol, a major lipid raft component, and (c) Rab proteins are key effects of endocytic traffic. We validated these three hits from our screen by targeted siRNA knockdowns, showing that knockdown of any of these three proteins dramatically reduced the PM localization of the raft TMD probe (Fig 11 A-B).

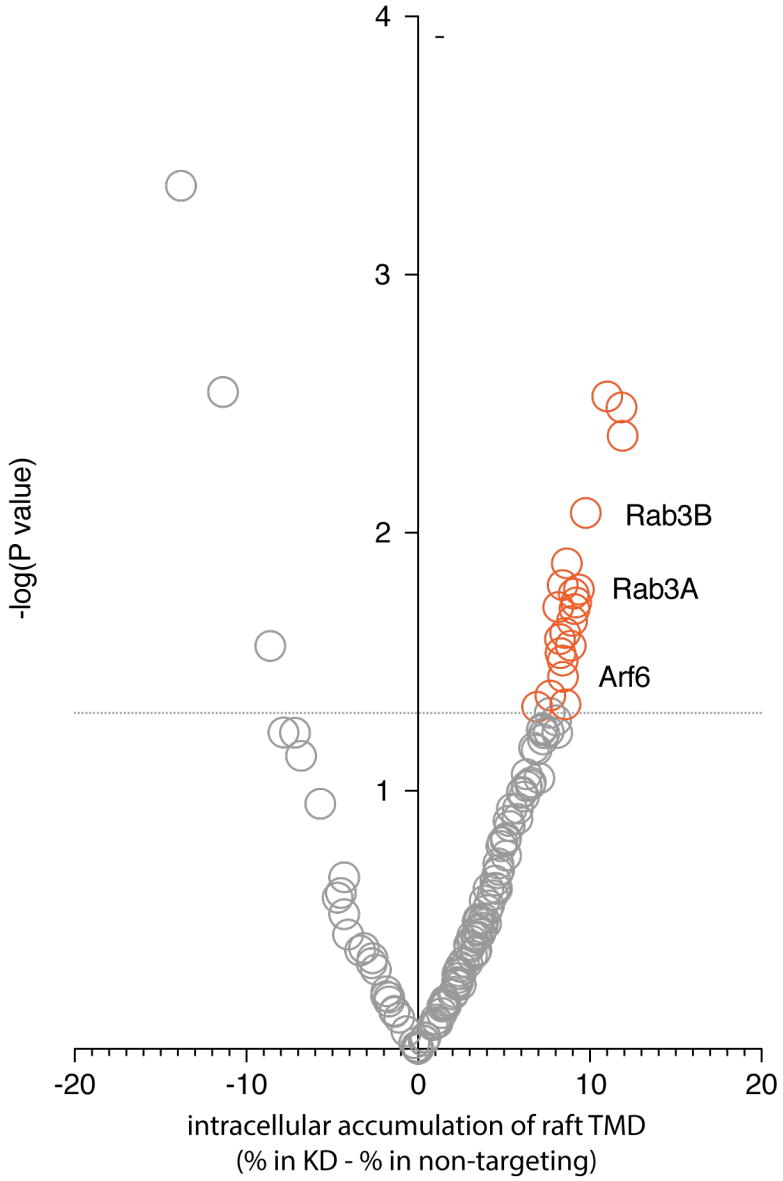


Figure 10 - Machinery for raft-mediated recycling. Volcano plot of the 150 proteins in the candidate library. x-axis is the change in intracellular accumulation of the raft TMD probe. y-axis is the -log of the p-value.

Table 5. Positive hits for putative effectors of raft mediated PM recycling.

Hit	p-value	Function
CLTCL1	0.01	Vesicle coat
RAB3B	0.01	Protein transport
AP4E1	0.01	Vesicle coat
WASF2	0.02	Cytoskeleton signaling
RAB3A	0.02	Protein transport
SYNJ1	0.02	Phosphatase
EPS15L1	0.02	Vesicle coat
CAMK1	0.02	Kinase
SYT1	0.03	Vesicle transport
ASAP2	0.03	Arf GAP
ADAM10	0.03	Protease
TSG101	0.03	Vesicle Trafficking
RAB5B	0.04	Protein transport
VCP	0.04	Membrane sorting
ARF6	0.05	Protein transport
EPN2	0.05	Endocytosis

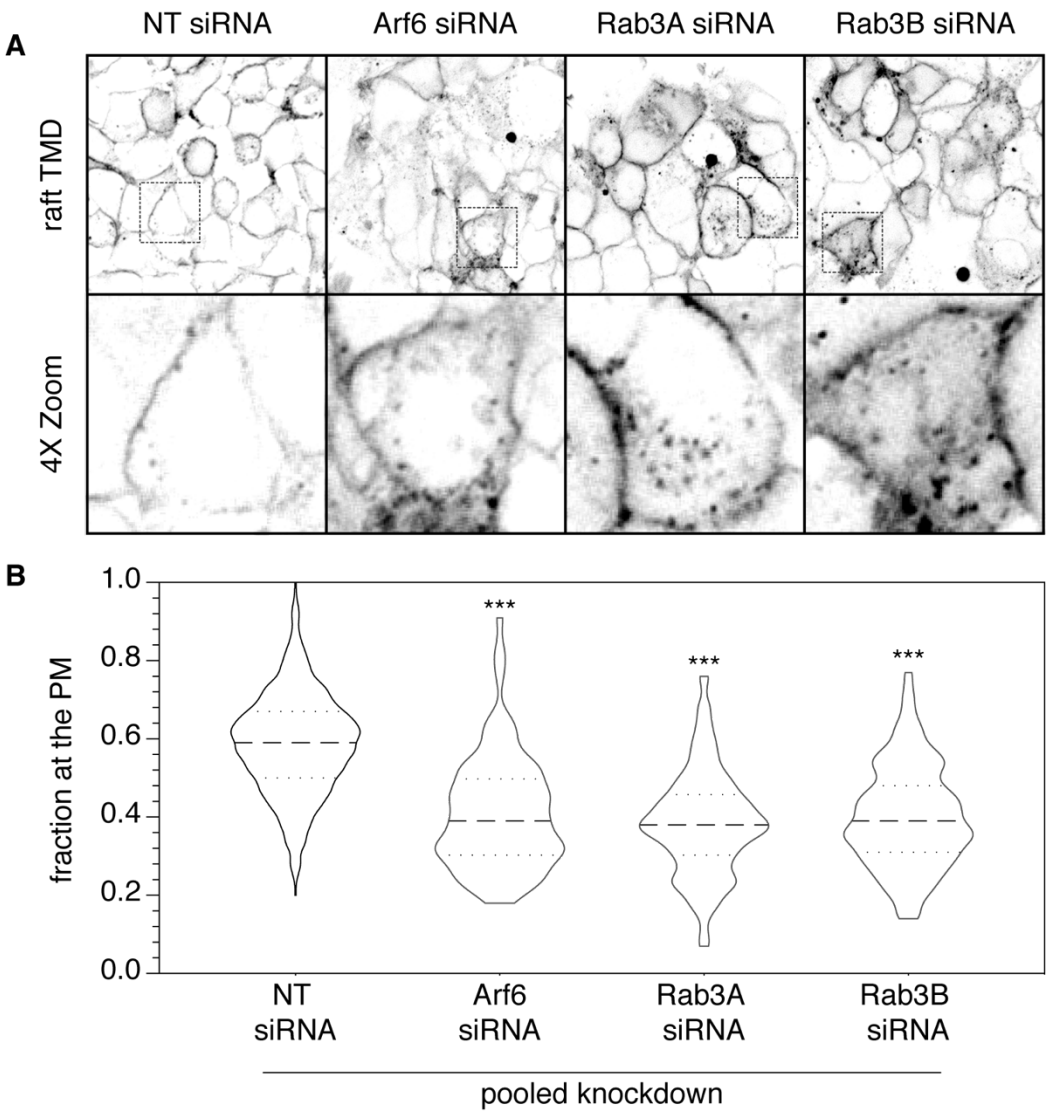


Figure 11. Hit validation. (A) Representative images of knockdown for 3 different hits in cells constitutively expressing raft TMD. Second row is a 4X zoom of the area marked by the outlined square. In all cases knockdown of the protein resulted in increased presence of raft TMD in intracellular vesicles. (B) Quantification of the fraction of raft TMD fluorescence at the PM, violin plot of 400-500 cells measured per knockdown. *** one-way ANOVA correcting for multiple comparisons relative to no-targeting (NT) siRNA.

4.3 Summary

Only one family of Rab-GTPases was identified in our positive hits, and two of the four isoforms of Rab3 were positive hits. For this reason, and because Rab-GTPases play a major role as regulators in other trafficking pathways, we decided to focus on validating and further characterizing the role of Rab3 in raft-mediated recycling. The other hits on the list provide useful insights into the plausible pathway that raft mediated recycling follows after reaching the late endosome. Two of the hits, Arf6 and SYT1, have been previously shown to interact with Rab3A (Pelletán et al., 2015; Schluter, Khvotchev, Jahn, & Sudhof, 2002) which suggest that they might all be players in the same pathway.

Chapter 5

Rab3 is a key component of the raft-mediated recycling machinery

5.1 Introduction

Rab3 is known to play a role in synaptic vesicle release and is thought to be expressed mostly in the brain. Therefore, first, we verified that Rab3 was expressed in our clonal cells and determined which of the four isoforms were present. Our cell line HEK-293 expressed all four isoforms, similar to the total human RNA control. Other common cultured cell lines also expressed at least two Rab3 isoforms, though each with distinct expression patterns (Fig. 12 A) The expression was confirmed using a Rab3 antibody to detect Rab3 in lysates of our parental cell line as well as each of the cell clones (Fig. 12 B).

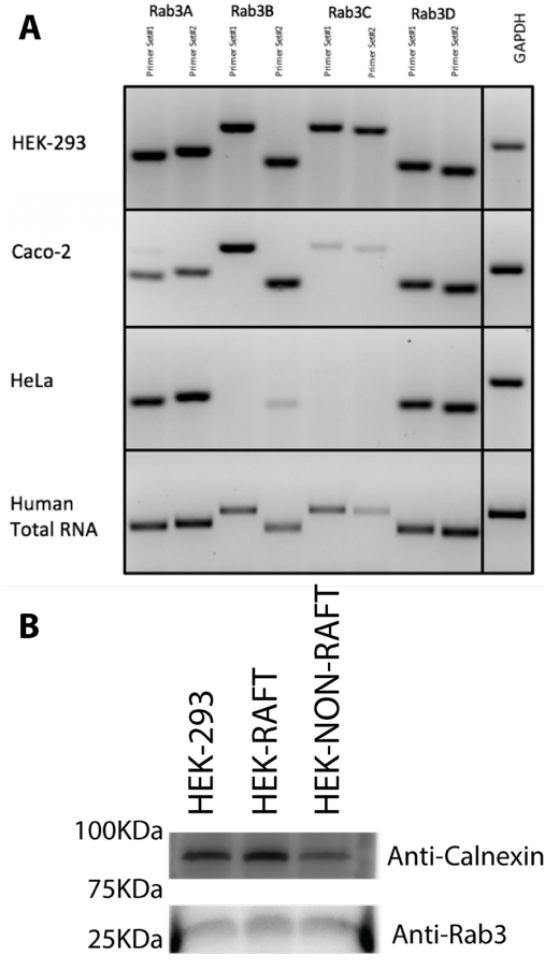


Figure 12. Rab3 A/B/C/D are expressed in HEK cells. (A) mRNA expression of all 4 isoforms of Rab3 in different cell lines (B) protein expression in HEK cells and clonal cell lines expressing raft TMD and non-raft TMD probes.

5.2 Results

5.2.1 Rab3 is an effector of raft-mediated recycling

As in our above-described experiments with the other Rab family members, I created plasmids expressing fluorescently tagged Rab3A and Rab3B isoforms and their dominant negative variants. As with Rab5 and Rab7 (Fig. 7), overexpression of GTP-locked versions of either Rab3A and Rab3B led to intracellular accumulation of raft TMD (Fig. 13A). When all four Rab3 isoforms were knocked down simultaneously, accumulation of raft TMD was particularly striking and highly significant (Fig. 13B-C). No effect on localization of the non-raft TMD was observed with any of the Rab3 perturbations (Fig 13B-C).

To identify the vesicles in which raft TMD accumulated, I immunostained cells treated with Rab3 siRNA. Raft TMD-containing vesicles were labeled by anti-Rab7 antibody, corroborating my previous finding that raft-mediated recycling vesicles originate from the late endosome (Fig. 14A). And just like we have seen before the number of intracellular vesicles increased when Rab3 was knock down and all these vesicles were stained by Rab7 antibody. Furthermore, to test if Rab3 and Rab7 were interacting in the native environment, I immunostained cells with Rab3 and Rab7 antibodies and imaged by super-resolved structured illumination microscopy (SIM). The resolution of SIM allows us to have a better picture of the small trafficking vesicles and detect if they are indeed in close proximity or even in some instances in the same vesicle. We quantified this effect by measuring the coefficient of colocalization (Fig.14B-C), showing that indeed Rab3 strongly colocalized with Rab7.

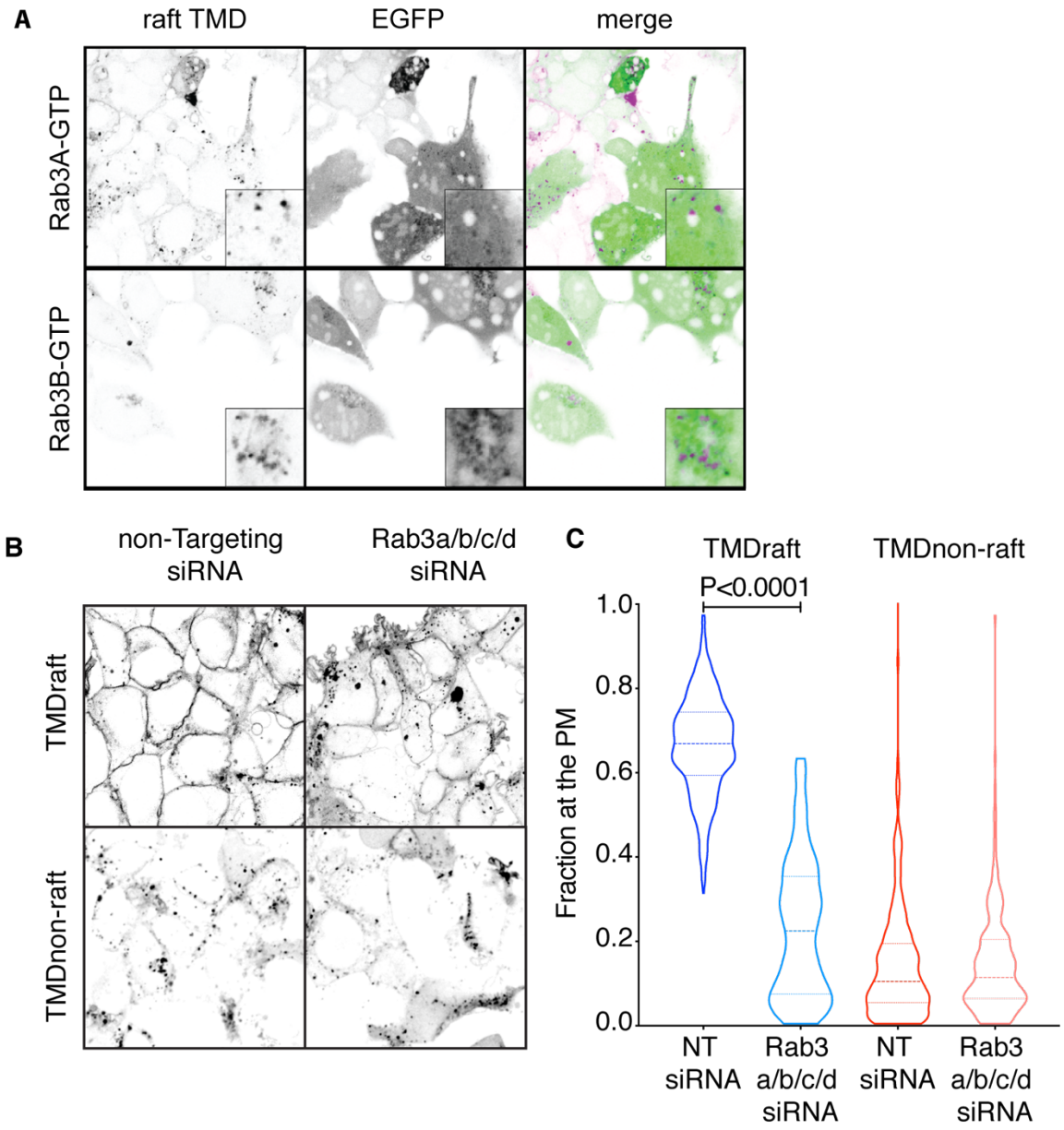


Figure 13. Rab3 is essential for raft-mediated recycling. (A) GTP-locked mutants of Rab3A and Rab3B were overexpressed in HEK cells expressing raft TMD, leading to accumulation of raft TMD in intracellular vesicles. (B) Knockdown of all 4 Rab3 isoforms (using siRNA) in cells expressing either raft TMD or non-raft TMD induced accumulation of raft TMD in intracellular vesicles. (C) Quantification of the fraction of raft TMD or non-raft TMD fluorescence at the PM. Violin plot of 250-300 cells measured per treatment. P value was calculated by t-test compared to NT siRNA.

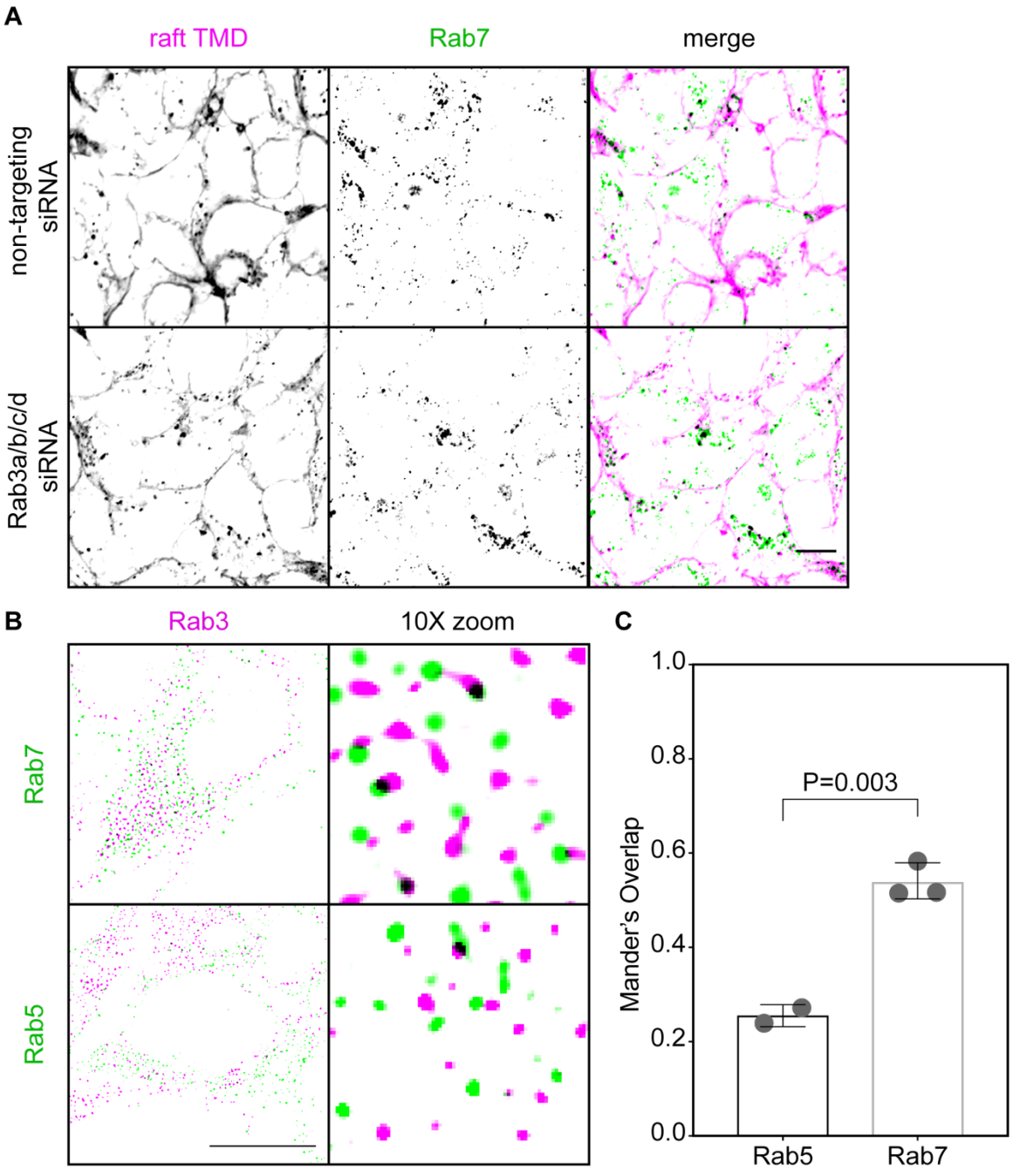


Figure 14. Rab3 vesicles associate with late endosomes. (A) Immunostaining of Rab7 in cells expressing raft TMD in which all 4 isoforms of Rab3 were knocked down using siRNA. Shows that the vesicles that accumulate raft TMD inside the cell are late endosomes. (B) Co-Immunostaining of Rab3 with either Rab7 or Rab5, images were taken using Structure illumination microscopy (SIM), revealing that Rab3 preferentially interacts with Rab7 compartments. (C) Quantification of the overlap between the two

co-immunostained Rab proteins using the Mander's coefficient calculation. Each dot represents the average of one experiment measuring 5-10 cells. P value was calculated using T-test.

5.2.2 Rab3 is palmitoylated and targeted to lipid rafts

Palmitoylation has been previously implicated as a strong determinant of raft affinity (ref). All four Rab3 isoforms have a C-terminal cysteine that is potential target for palmitoylation. To test whether they were indeed palmitoylated, I performed acyl-biotinyl exchange (ABE) and compared to other Rab GTPases. I observed that Rab3A is indeed palmitoylated to a much greater extent than any of the other endosomal Rabs (Fig.15 A). None of these showed detectable palmitoylation levels, except for Rab7, which I have shown interacts with Rab3. Finally, I observed that Rab3 is present in detergent resistant membrane fractions, strongly indicating that it interacts with lipid rafts (Fig 15 B-C).

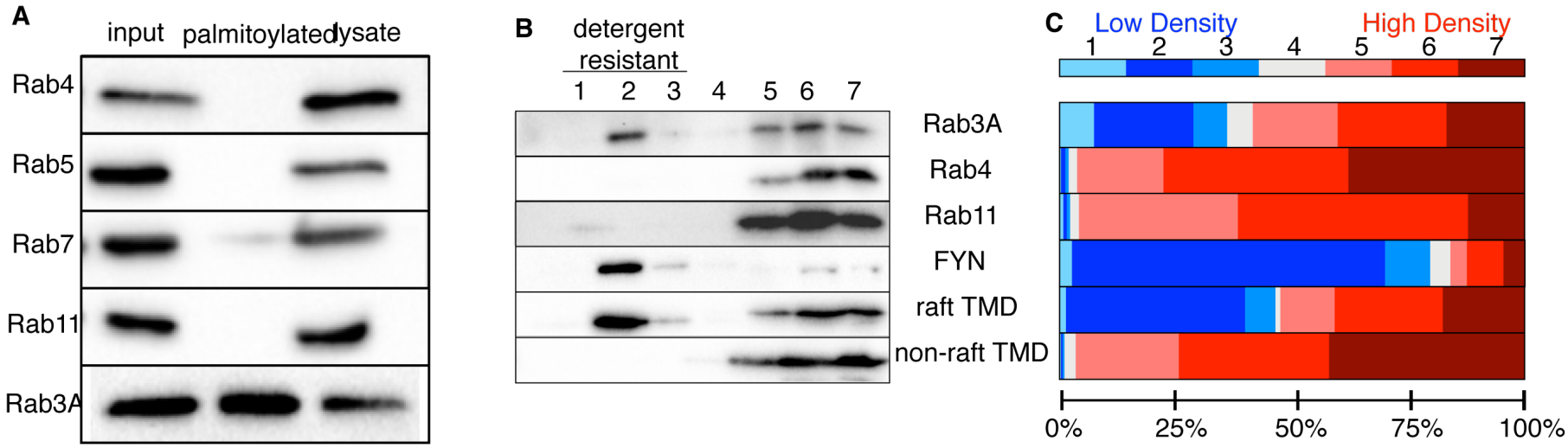


Figure 15. Rab3 is palmitoylated which may mediate Rab residence in ordered membranes. (A) Western blot of an acyl-biotin exchange experiment to detect palmitoylated proteins. Rab proteins of the endocytic system were immuno-blotted for expression in HEK whole cell lysate and for palmitoylation. (B) Western blot of all fractions separated by density, proteins remaining in detergent resistant membranes will be present at lower densities than soluble proteins. (C) Quantification of the percentage of each protein present in each fraction of the density gradient.

5.2.3 Raft-partitioning proteins require Rab3 for proper PM localization

To test the role of Rab3 in trafficking of full-length proteins, I expressed several such proteins in HEK cells and used siRNA to knock down all four isoforms of Rab3. For several known raft-preferring proteins, including GPI-GFP and the EGF-receptor, PM localization was strongly dependent on Rab3 (Fig. 16). Knockdown led to accumulation of these proteins in intracellular puncta, as for the raft TMD probe. Proteins not partitioning to raft domains, like the transferrin receptor (TfR) were unaffected by Rab3 KD.

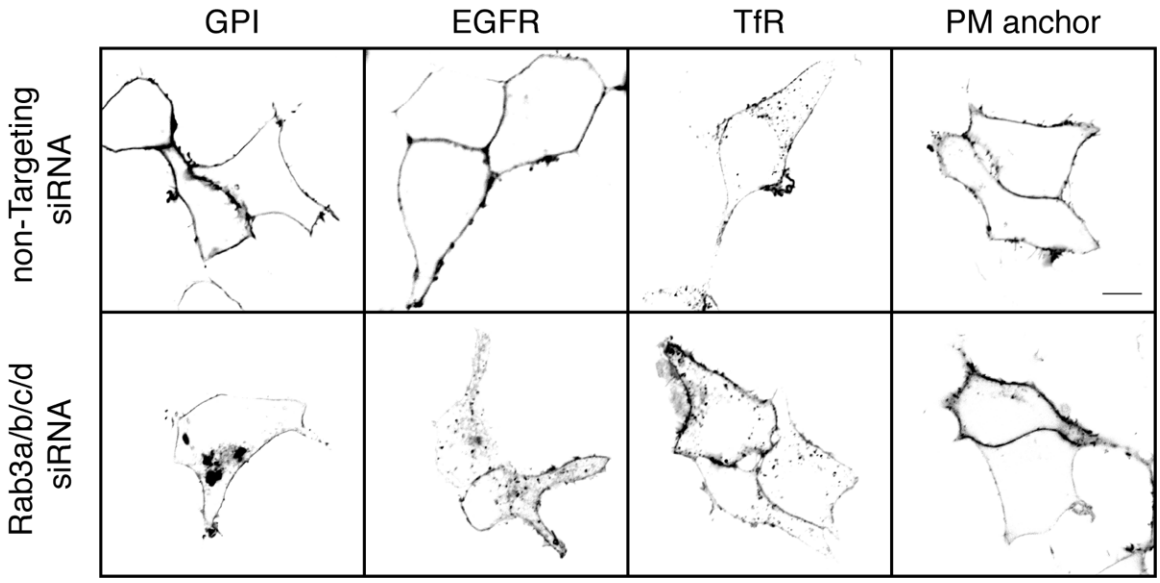


Figure 16. Full length proteins utilize raft mediated recycling route. All isoforms of Rab3 were knockdown using siRNA in cells expressing GPI anchored GFP, full length EGF and Transferrin receptor tagged with GFP and the membrane binding domain of KRas bound to GFP, a non-targeting siRNA was used as a negative control.

5.3 Summary

All four isoforms of Rab3 are known to play a role in some type of exocytosis in specialized cells, mostly cell types whose function requires increased secretion. I show that Rab3 is also expressed in other cell types to various degrees and that they play a role in recycling components from the late endosome to the PM. I also show that the proteins that follow this pathway have a preference to reside in membrane microdomains know as lipid rafts. And that Rab3 itself resides in lipid rafts, and that this association might be due to a post-translational modification, palmitoylation.

Chapter 6

Concluding remarks and future directions

6.1 Future Directions

In order to understand the extent that the raft mediated recycle pathway plays in cell trafficking I created a Rab3 knock down cell line using CRISPRi. Just like when I used siRNA to decrease protein expression I can see that this cell line is unable to recycle raft TMD (Fig. 17) This cell line will allow me to investigate what other proteins require this pathway to recycle to the PM. And how it is implicated in lipid trafficking and homeostasis. Having this resource allows for further investigation of the cargo of this pathway both in protein by protein specific interrogation to see if the PM localization of a protein of interest is affected. And also, in a more systematic way using comparative proteomics and lipidomics to see how inhibiting this pathway changes the composition of the PM.

The Rab3 KD cell line will also allow us to test directly which of the isoforms plays a major role in raft mediated trafficking by expressing each isoform in the cell line and seeing if it rescues the trafficking it the raft TMD, or if they indeed are completely redundant and able to compensate for one another. It also allows us to add back different mutants of Rab3 protein to test the role that GDP bound mutants plays compared to GTP locked. It will also allow us to test if palmitoylation is necessary for its function and if this modification is indeed the way Rab3 is targeted to lipid rafts.

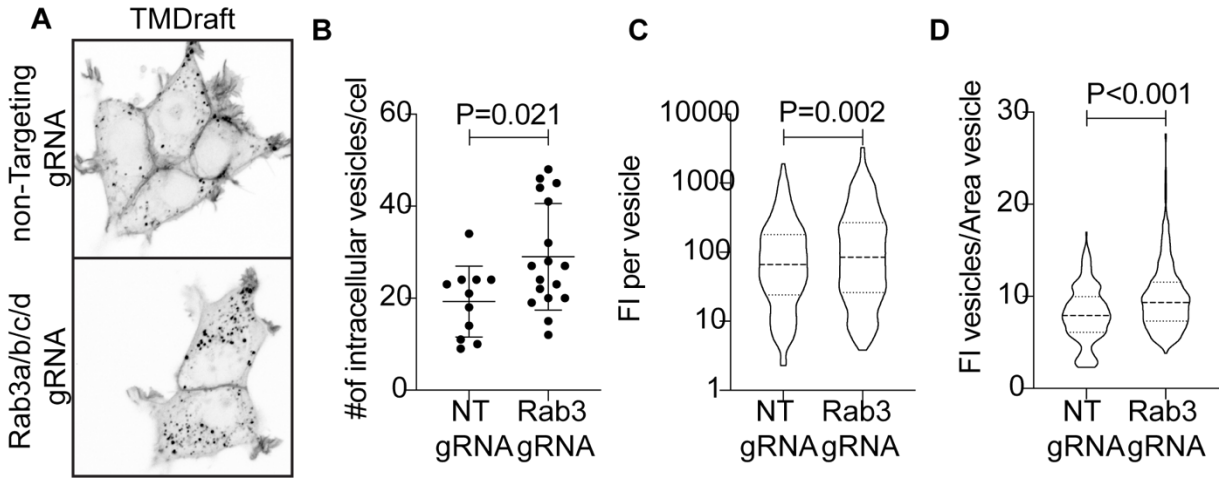


Figure 17. Rab3 is a key player of raft mediated recycling. (A) CRISPRi cell line for knockdown expression of the four isoforms of the Rab3 family in cells constitutively expressing Raft TMD. (B) Quantification of the number of intracellular vesicles per cell, each dot represents one cell measured. The P value was calculated with a T-test comparing Rab3 KD with a non-targeting gRNA. (C) Quantification of the fluorescence intensity per intracellular vesicle, the violin plot is the distribution of each vesicle measured. The P value was calculated with a T-test comparing Rab3 KD with a non-targeting gRNA. (D) Quantification of the fluorescence intensity per vesicle normalized to the vesicle area, the violin plot is the distribution of each vesicle measured. The P value was calculated with a T-test comparing Rab3 KD with a non-targeting gRNA.

6.2 Discussion

Rab3 is only one player of this pathway and as mentioned before vesicle trafficking requires multiple steps that involved a number of specialized proteins, therefore the pathway most likely involves various players. I have a list of other hits that can be validated, some of which are known to interact with Rab3. Another way we can

continue to discover the players in this pathway is to test how other proteins that are known to interact with Rab3 affect raft-TMD localization.

In the data presented here I show that the raft and non-raft TMD probes traffic to the late endosome after endocytosis from the PM, that after the late endosome their trafficking route differs from one another. And that Rab3 is required to retrieve the raft preferring proteins from the late endosome, and that this retrieval is necessary for the protein to recycle back to the PM. From here the pathway can be either straight to the PM or through other secretory organelles (Golgi), trafficking pathways in the cells are known to be interconnected, there is a strong possibility that this pathway may share machinery from other pathways. Even for it to be connected to other pathways, one such possible pathway can be the retromer, which also originates from the late endosome to return cargo to the Golgi for their secretion. All of these scenarios require further investigation. From what is known of how Rab3 functions in the brain we could infer that Rab3 travels with the vesicle to the PM and aids in recruit the machinery necessary for fusion as with synaptic vesicles, but this too requires further testing.

Throughout the process of investigating the raft mediated recycling pathway I probed and interfered with the endocytic pathway in many ways (inhibitors, protein overexpression, etc.). In all instances I saw first-hand how important the pathway is for cell fitness, as any perturbation eventually resulted in cell death. When I tried to knock out all four isoforms of Rab3 it was not unexpected that this was not tolerated by the cells, and why I chose to continue with a knockdown system instead. While this is not the perfect system and some of the perturbations are not as striking in populations studies it does provide a good way to test the role of Rab3 in trafficking.

So far, we have tested protein-based probes and some full-length proteins, which are a small representation of the number of cargos that could utilize this pathway to maintain proteins and lipids at the plasma membrane. We inferred from the association of the probes with the lipid rafts that the lipid components of this membrane domains are also trafficked on the same way, it is hard to test each lipid component in a cell-based assay because adding fluorescent tags changes their biophysical properties and behavior, but lipidomics of the plasma membrane can be done to see what is changing when we perturb lipid raft mediated recycling.

6.3 Summary of conclusions

Intracellular trafficking has generally been elucidated one protein or protein family at a time. However, it is probably more accurate to think of trafficking as a collective behavior of not just one protein, but rather a collection of proteins and lipids. Lateral segregation (sorting) of membrane lipids and proteins based on their biophysical properties is a reasonable way to differentiate between components of different organelles. Here I have demonstrated that raft affinity is a determinant for PM maintenance.

These results identify a novel mechanism for intracellular protein sorting and define a physiological role for lipid rafts in cells. Furthermore, I have identified some of the key molecular machinery mediating lipid raft recycling and defined a novel function for Rab3. These findings fill a major gap in knowledge in the trafficking field and demonstrate that, like in other cellular processes, the cell has more than one way to accomplish a task. Our findings allow us to update our graphical model of the involvement of lipid rafts in membrane trafficking. (Fig. 18)

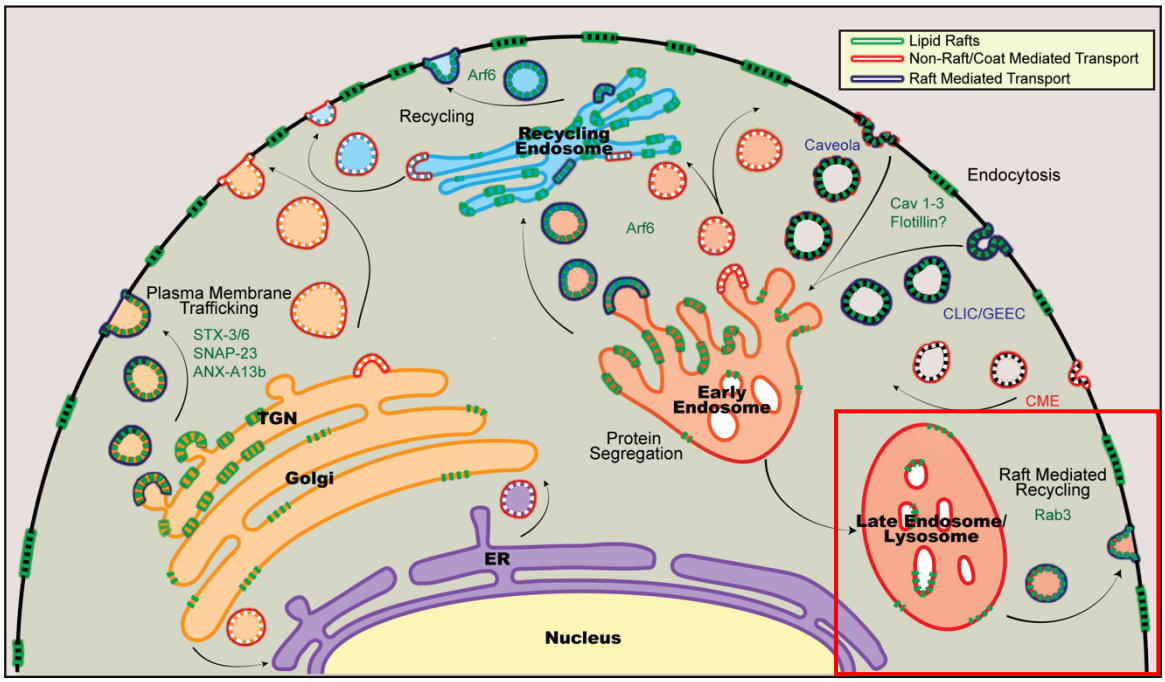


Figure 18. Involvement of raft domains in membrane traffic.

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