# Rapid endosomal recycling

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Chapter 6

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Additional information is available at the end of the chapter

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**Abstract**

Peripheral membrane proteins are endocytosed by constitutive processes of membrane invaginations, followed by internalization driven by diverse endocytic machinery available at the cell surface. It is believed that after endocytic uptake, cargo proteins proceed either through the endosomal recycling circuit of the cell or travel toward late endosomes for degradation. In this chapter, we analyzed trafficking of seven cargo molecules (transferrin receptor, fully conformed MHC-I, non-conformed MHC-I, cholera-toxin B subunit, CD44, ICAM1, and G-protein-coupled receptor Rae-1) known to use the distinct endocytic route. For that purpose, we developed the software for multicompartment analysis of intracellular trafficking. We demonstrate that all endocytosed molecules are rapidly recycled and propose that the rapid recycling is a constitutive process that should be considered in the analysis of intracellular trafficking of peripheral membrane proteins.

**Keywords:** rapid endosomal recycling, clathrin-independent cargo, endosomal recycling, endosomal trafficking, kinetic modeling of endosomal trafficking, transferrin receptor

## 1. Introduction

Endocytosis is an essential cellular function maintenance of the membranous system and plasma membrane (PM) associated functions, including uptake of nutrients and extracellular material, cell communication and information processing, motility, adhesion, and cell division (reviewed in [1–7]). Endocytic uptake occurs either by ligand binding to cell surface proteins (receptor-mediated endocytosis) or by uptake of extracellular fluid by membrane invaginations (fluid-phase endocytosis) [3]. Endocytosis is initiated by cellular proteins that change PM lipid composition and by the assembly of a series of cytoplasmic proteins,

known as endocytic machinery, which causes membrane deformation and assists pinching off membrane invaginations into endocytic carriers. The best characterized is cytoplasmic protein clathrin that assembles and forms clathrin coat which initiates the rapid development of endocytic carriers, known as clathrin-dependent endocytosis (CDE). Endocytic carriers may be developed without the assistance of the clathrin, known as clathrin-independent endocytosis (CIE), although it is assisted by cellular proteins such as caveolin (known as caveolin-dependent endocytosis) or flotillin (flotillin-dependent endocytosis). Pinching off endocytic carriers is also facilitated by several cellular proteins, including cellular guanosine-triphosphatase (GTPase). Endocytic uptake that requires the dynamin is known as dynamin-dependent endocytosis (DDE), and the endocytic uptake which does not engage dynamin is known as dynamin-independent endocytosis (DIE).

In addition to the endocytic uptake initiated by ligand binding, PM is constitutively endocytosed with the dynamic unique for each cell. Depending on cell type, the whole cell surface is internalized one to five times in an hour [3]. The dynamic of the constitutive uptake, thus, is a cell adaptation to the metabolic and growing conditions. Constitutive endocytic uptake of PM is a part of cellular physiology required for maintenance of PM and membranous organelle composition. PM is internalized into endocytic carriers, which upon endocytic uptake coalesce and together with endocytic carriers derived by receptor-mediated endocytosis form early endosomes (EEs). EEs are highly dynamic intracellular compartments that grow in size, migrate along cytoskeleton, fuse with each other, mix and sort membrane content, mature, and ultimately deliver the membrane content to lysosomes for degradation [1, 8]. Along the endocytic pathway, membranes change composition and form membrane domains, which at some stage of endosomal maturation can form subcompartments [1, 2, 7]. Maturing EEs form larger organelles, which extensively sort membrane cargo, known as sorting endosomes (SEs), by process of endosomal conversion transform into late endosomes (LEs). LEs are a highly dynamic network of membrane domains that also mix and sort membrane cargo and deliver it either toward the *trans*-Golgi network (TGN), cell surface (exocytosis), or lysosomes for degradation [1, 7, 9].

Along the entire endosomal pathway, endosomal membranes develop recycling domains to generate recycling carriers that return membranes back to the cell surface (reviewed in [10–12]). As endocytic uptake, endosomal recycling may be initiated and regulated by a set of cellular proteins representing the recycling machinery and may occur constitutively. At the stage of EEs, a majority of the endocytosed membrane is returned to the cell surface from tubular EEs that directly deliver recycling carriers to PM or transform into the agglomerate of recycling domains near the cell center, known as the endosomal recycling compartment (ERC). EEs and the ERC return the majority of internalized membranes, although recycling may also occur from LEs [13], and only 3–5% of internalized membranes are delivered to lysosomes. Although it is considered that endosomal route through EEs and the ERC represent the endosomal recycling circuit, and LEs represent the feeder system that delivers cargo into degradation [1], it appears that recycling occurs from LEs and the endosomal recycling route can be divided into EE and LE recycling circuits [13].

Constitutive endosomal uptake and recycling are processes utilized by the cell to regulate PM and endosomal organelle composition and represent a fundamental mechanism for cellular adaptation to the metabolic activity and environmental conditions. Maintenance of the

membranous system consumes ~30% of cellular energy [14] and, thus, it is of particular interest for the cell to make these processes energetically efficient. The delivery of endocytosed membranes and membrane cargo along the endocytic pathway should be aligned with the cellular physiology requirements for endosomal compartmentalization, and all membranes and membrane cargo that do not need to enter the distal parts of the endosomal pathway should be returned back to the cell surface as early as possible to minimize cellular energy consumption. Therefore, for a substantial part of an internalized PM, it is essential to be returned quickly after endocytic uptake. This process of membrane return should occur rapidly after both ligand-initiated and constitutive endocytic uptake and may be called rapid recycling.

The term rapid recycling is not clarified in the literature. Although the term rapid recycling can also be assigned to the recycling processes that are characterized by the very fast delivery of membranous content to the PM at any stage of the endocytic tract, we assign the term rapid recycling to a part of the recycling circuit that is activated very early after endocytosis. In this chapter, we used available data from the literature, our experimental data, and kinetic modeling to demonstrate that rapid recycling is a significant constitutive part of post-endocytic itinerary irrespective of the way of membrane endocytic uptake.

## 2. Plasma membrane dynamics

Early studies on endocytosis suggested that the PM is highly dynamic and the whole PM is internalized one to five times in an hour [2]. Studies using fluorescent lipid analogs demonstrated that the PM system is extremely dynamic and that the half-time for membrane turnover could be as short as 5–10 min [15]. Constitutive endocytic uptake occurs by clathrin-coated pits and by clathrin-independent mechanisms (reviewed in [5, 6]). Thus, it is reasonable to assume that the constitutive uptake does not occur at the entire PM with the same rate. In addition to the wide range of endocytic machinery available in the cell, the rate of PM constitutive uptake would also depend on membrane composition at the site of development of endocytic carriers and attachment of PM to the actin network. For example, requirements for membrane deformation and transformation into an endocytic carrier will be different at lipid-organized membrane microdomains (i.e., lipid rafts) than at more fluid parts of the PM.

Much understanding of the kinetics of the PM uptake came from the studies of CDE cargo, mostly the transferrin receptor (TfR). In general, internalization kinetics of CDE cargo molecules demonstrated that PM uptake at the segments which involves clathrin-coated pits (coat-dependent endocytic uptake) is very fast [4, 15] and occurs with the rate which is in the range of 0.20–0.50 $min^{-1}$ [16–19], although lower [20] and higher [19] rates were determined. Kinetics of the endocytic uptake of CDE cargo, however, does not reflect the average rate of the constitutive uptake of PM. Namely, the coat-dependent route accounts for 40–50% of the constitutive endocytic uptake [5], although recent evidence suggests that at least 95% of cellular endocytic uptake is based on clathrin-coated pits [21] and that different cell surface proteins can be sorted into distinct clathrin-coated pits [2, 4]. On the other hand, the kinetic and physiology of the constitutive endocytic uptake that does not involve clathrin coats (coat-independent pathways) is poorly analyzed. It appears that the rate of the constitutive endocytic uptake is much lower

than in the coat-dependent pathway [22, 23]. However, these rates are mostly determined as the uptake of soluble enzymes or fluorescently labeled molecules and, thereby, may represent the rate of fluid-phase uptake, which is one smaller segment in the coat-independent pathway. Very little quantitative analysis was performed by measuring the constitutive endocytic uptake of coat-independent PM cargo proteins. Even studies based on the incorporation of fluorescent lipid analogs (i.e., sphingomyelin), which are an excellent tool to determine PM dynamics and demonstrated a high rate of the PM uptake [15], did not accurately measured the uptake of the entire PM, but rather PM segments that incorporated the lipid analog.

In general, it is difficult to measure the rate of PM uptake. In fact, most studies determined the internalization rate (IR) which represents the difference between the endocytic rate (ER) and the recycling rate (RR). Thus, any measurement that may account recycling does not represent the rate of PM uptake (ER) but rather IR. Given that the recycling may occur very early after endocytosis (i.e., one or two min. after initiation of the endocytic uptake), any measurement that is longer than 2 min, thus, potentially determines the IR and not the ER. Since it has been shown that membranes labeled with fluorescent lipid analogs rapidly recycle with the high rate, in the range of 0.17–0.70 $min^{-1}$ [15, 18], it can be estimated that the rate of PM uptake must be higher. Thus, understanding the dynamics and activation of the rapid recycling mechanism is essential for understanding the turnover of the PM. Similarly, to understand the cellular physiology of any peripheral membrane protein, including feedback mechanisms that determine its intracellular distribution and consequently function, it is essential to construct its intracellular itinerary, which includes endocytic uptake, inter-endosomal trafficking, and endosomal recycling. Therefore, in addition to PM dynamics, it is essential to understand quantitative aspects of the cellular physiology of the endosomal system, particularly the EE system.

## 3. The early endosomal system: a brief overview

The early endosomal (EE) system is the complex network of membranous vesicular and tubular structures that continuously exchange cargo and form membrane domains with different functions [1, 8]. The endosomes undergo fusion and fission reactions which shape the number and size of organelles. During these reactions, the cargo is sorted either into tubular domains for recycling to the PM and TGN or into intraluminal vesicles for degradation. Several hundreds of individual endosomes form a dynamic network and create funnel-like system [8] in which endocytosed cargo progressively flow from small endosomes at the cell periphery to large endosomes in the cell center until Rab5-positive EEs convert into Rab7-positive LEs [7, 24]. Recent studies demonstrate that the EE system can be subdivided into at least two stages: proximal comprised of pre-EEs and the distal comprised of EE/SEs [8, 25–27].

### 3.1. Pre-EEs

We consider pre-EEs as subcortical endosomes which are positive for Rab5, as all EEs, and devoid of early endosomal antigen 1 (EEA1) and phosphatidylinositol-3-phosphate (PI3P) [8, 27]. These endosomes accept CDE cargo, such as TfR [26], epidermal growth factor receptor (EGFR) [2, 25], beta-2-adrenergic receptor (B2AR) [28], and luteinizing hormone receptor (LHR) [27]. A subpopulation of Rab5-positive pre-EEs recruit APPL1 (adaptor protein, phosphotyrosine interaction,

PH domain and leucine zipper containing 1) [29, 30] and represent a stable sorting station, not only intermediate in EE maturation [26]. However, APPL1 endosomes are not the only entry site for clathrin-coated pits, since a significant fraction of clathrin-coated pits do not acquire APPL1 following uncoating [29] and a substantial fraction (approx. 40%) of internalized Tf does not colocalize with APPL1 or EEA1 at early times after internalization [26].

### 3.2. Early/sorting endosomes

EEs are not the homogenous population of endocytic compartments which accept all internalized cargo without discrimination, but instead are comprised of distinct populations regarding mobility and maturation kinetics [31]. EEs represent a broader profile of tubular and vacuolar compartments that are characterized by the presence of PI3P, EEA1, and Rab5 [31] and form a dynamic network. Endosomes in the central part of this network develop vacuolar and tubular domains [32], undergo fusion and fission reactions [8], and represent earlier stages of endosomal maturation. It also includes dynamic endosomes [31] and APPL1+EEA1 endosomes [29, 30]. Maturation of EEs involves either generation of the tubular endosomal domain which recycles cargo to PM or vacuolization and formation of intraluminal vesicles which ends up with conversion into LEs [24, 31, 32].

The vacuolar domain of EEs retains cargo destined for degradation and sort it into intraluminal vesicles whereby vacuolar EEs become multivesicular endosomes. Limiting membranes of vacuolar EEs generate tubular-sorting endosome (TSE) [32] or tubular endosomal network (TEN) [9] that sorts plasma membrane recycling proteins either into recycling carriers or develop into the ERC. TSE/TEN also sort lysosomal membrane proteins (Lamp1, Lamp2, and CD63), sortilin and M6PR into LEs or TGN [32]. Also, limiting membranes of vacuolar EEs, just before their maturation into LE vacuoles, can develop the endosome-to-TGN transport carriers (ETC) specific for retrograde transport of lysosomal proteins [33]. It has been shown that several CDE cargo molecules can pass through the same EE vacuoles but exit this organelle through different recycling tubules, i.e., the TSE/TEN and ETCs [33].

Live cell imaging [31] and fluorescence resonance energy transfer (FRET) analysis [32] demonstrated the existence of two kinetically distinct populations of EEs, dynamic and static. Dynamic EEs is a smaller peripheral subpopulation of EEs that transfer cargo into LEs with the fast rate, whereas static EEs are multivesicular perinuclear endosomes that undergo an EE-to-LE transition as the latest stage of EE maturation [31, 32]. Static EEs are a pleomorphic structure composed of large vesicles and thin tubular extensions that have membrane invaginations and develop multivesicular appearance [32], sort CDE and CIE cargo, and transport CIE cargo by MICAL1-positive TREs or CDE cargo by Rab11-positive carriers to the ERC or the cell surface [34].

### 3.3. The endosomal recycling compartment

The endosomal recycling compartment (ERC) is membranous tubulovesicular organelle organized in the pericentriolar region [10, 11]. The ERC is constructed around the MTOC as a network of both partially connected, and individual vesicles and tubules organized [10, 34] which is distinct from membrane-bound tubular recycling endosomes (TREs) [34]. ERC is spatially confined within the Golgi, whereas EEs, LEs, and Ly are excluded from inside the Golgi [35].

## 4. Endosomal recycling

Early studies of membrane recycling demonstrated that of all endocytosed membrane 95% is eventually recycled, and only 5% is targeted to lysosomes [3]. Most of the knowledge about endosomal recycling routes were generated using conventional assays based on redistribution of receptors, mostly from the TfR (reviewed in [11]). In contrast to CDE cargo proteins, the recycling route was not established or is poorly characterized for many proteins that are endocytosed by the CIE. The best characterized is the recycling route of major histocompatibility class I (MHC-I) proteins [11], although recycling routes of several CIE cargo proteins have been described in the last decade [6, 11, 36].

### 4.1. Fast and slow endosomal recycling

Recycling route, recycling rate, and recycling efficiency are well established for TfR due to the availability of an excellent tool appropriate for radioactive, chemical, or fluorescent labeling [15, 16]. Recycling of TfR occurs by iterative fractionation during EE trafficking [10, 11, 37] and the recycling kinetics is typically biphasic, with the initial fast and the later slow component [17]. Thus, TfR recycling route is usually divided into two steps: a fast or direct route that occurs from EEs, sometimes called rapid recycling route [11], and slow or indirect route that occurs from the ERC [10–12, 19, 37]. Recycling of TfR from EEs requires the function of Rab4 [7, 10, 11, 18], whereas recycling from the ERC requires the sequential function of multiple regulators including Rab5, Rab11, Rab8a, their effectors (Rabenosyn-5, Rab11-FIP2, and MICAL-L1, respectively), and EHD proteins [34]. TfR can be recycled from both static and dynamic EEs, including those in the process of Rab5 and Rab7 conversion [31]. Some cargo proteins, such as G protein-coupled receptors (GPCRs), require specific sequence and multiple interacting proteins for recycling [28, 38], whereas many proteins may recycle without any sequence and interaction requirements [10, 39], as a part of the bulk membrane flow. Sequence-dependent recycling occurs via tubular microdomains that are distinct from tubular domains that mediate the bulk recycling, even at the same endosome [40].

Using fluorescent lipophilic dyes, Hao and Maxfield [15] demonstrated rapid recycling endosomal membranes very early after endocytosis. They showed after 2 min of pulse internalization of NBD-SM that 30–60% of membranes is rapidly returned (recycled) with very high rate (0.35–0.70 $min^{-1}$), indicating very early activation of the recycling mechanism. Based on the kinetics of fluorescent dyes trafficking, they concluded that larger endocytic compartment than primary endocytic vesicles is involved in rapid recycling. In addition, a similar study showed rapid and extensive (with similarly high rate) mobilization of the significant fraction of membranes not only in the very early stage of endosomal flow but also from all later stages [39]. Thus, rapid and extensive engagement of membranes into recycling is a general property of the endosomal system, and the exchange of membranes between endosomes and PM is more extensive than it can be derived from studies using single membrane receptor.

### 4.2. Rapid endosomal recycling

Recent studies using live-cell imaging and total internal reflection fluorescence microscopy (TIRFM), suggest that recycling mechanism may be activated very early in the endocytic tract,

at the stage of pre-EEs or very early endosomes (VEEs). The TIRFM study of GPCRs recorded rapid recycling events as exocytic puffs that appeared at the cell surface 2–3 min after internalization [41], and live cell imaging studies demonstrated that EEA1- and PI3P-negative endosomes and APPL1-positive endosomes represent the very early recycling compartment [27, 40].

Although most of knowledge about recycling was constructed on studies of TfR recycling using radiolabeled or fluorescent ligand (Tf), uncovering the very early recycling step was difficult, because all assays were based on the quantification of the ligand release from the cell as an indication of recycling. However, the release of Tf from TfR only occurs when TfR reaches sufficiently acidic compartment which converts holo- into apo-Tf. Thus, it was long believed that CDE cargoes could recycle back to the cell surface through a pathway that requires Rab4 and Rab35 [11]. Nevertheless, recent studies demonstrated that TfR recycling might occur very early in the endocytic tract, from APPL1 endosomes, downstream APPL1-EEA1 endosomes which receive CIE cargo, and from EEA1 endosomes [8, 26]. The TIRFM study of GPCRs also recorded coincident puffs of fluorescent Tf-labeled receptors, indicating that the earliest recycling of TfR occurs without the release of apo-Tf [41]. These rapid recycling events were invisible by conventional assays, and thus rapid recycling processes were underestimated. The TIRFM imaging enabled visualization of very rapid recycling vesicles, since these vesicles contain endocytic cargo at relatively high concentration, whereas vesicles that mediate slower pathways of recycling or biosynthetic insertions contain cargo at a significantly lower concentration which cannot be detected by the TIRFM imaging. Most evidence for rapid recycling can be derived from studies on the post-endocytic itinerary of G protein-coupled receptors (GPCRs). Activated beta-2 adrenergic receptor (B2AR), a typical representative of the largest GPCR family, is endocytosed by the dynamin-dependent CDE mechanism [28, 38]. Internalized receptors rapidly recycle back to the cell surface in vesicles that concentrate internalized receptor, which recently enabled visualization of the rapidly recycled receptor by the TIRFM microscopy [41]. Also, during each cycle, a fraction of receptors is directed into EE/SEs and either to the ERC from which they are slowly returned to the cell surface or to lysosomes where they are degraded [28, 38]. Not all GPCRs follow the same endocytic itinerary. For example, follicle-stimulating hormone receptors (FSHRs) follow the same route as B2AR, whereas the majority of LHR and δ-opioid receptor (δOR) are targeted from EEs into lysosomes and degraded [27]. However, both types of receptors recycle from EEA1- and PI3P-negative small very-early endosomal (VEEs)/pre-EE compartment [27].

## 5. Analysis of rapid recycling by kinetic modeling

To analyze rapid recycling, we used kinetic modeling approach, which is based on the accurate measurement of cell surface kinetic and construction of the intracellular itinerary using the software which enables calculation of dynamic distribution through multiple compartments of endosomes [13, 42].

### 5.1. Selection of peripheral membrane cargo molecules

It is becoming clear that membrane proteins cannot be put into two categories (CDE and CIE cargo) but instead classified into broader spectrum regarding engagement of endocytic machinery, kinetics of the endocytic uptake, and post-endocytic itinerary [6]. Thus, we examined endocytic

itinerary of seven membrane cargo molecules (**Figure 1**) that have been shown to use the distinct endocytic route and that had a distinct post-endocytic itinerary. The route of TfR and GPCRs, CDE cargo proteins, is well defined [4, 9, 43] and involves rapid recycling [15, 27, 41]. Routes of the six CIE cargo molecules (**Figure 1**) are relatively well characterized, but their very-early post-endocytic itinerary is mostly unknown. Internalization pathways of two of these cargo molecules, GM1/CTxB and antibody-clustered GPI-anchored protein (GPI-AP) Rae1-γ [5, 44], require the activity of dynamin (DDE) [45]. These two cargo molecules may not only enter the cell via caveolae but also may be internalized DIE pathway which is regulated by the small GTPase Cdc42 [45]. The Cdc42-regulated pathway, also known as CLIC/GEEC pathway, is controlled by the small GTPase Arf1 and is used for fluid-phase uptake [5, 6]. Constitutive internalization pathway of three membrane proteins (fully-conformed MHC-I molecules, CD44, and ICAM1) is regulated by small GTPase Arf6 (known as Arf6-regulated pathway) [43, 46, 47]. One membrane cargo molecule (empty MHC-I) is internalized by the CIE mechanism, but dynamin requirement and small GTPase regulation are unknown [13, 48].

Irrespective of the way of entry into the cells, cargo molecules have distinct itineraries in the endosomal system upon endocytosis [43] and can be classified into six types according to known endocytic routes. *Type A cargo* proteins are long-lived, travel along the recycling route, and in EE/SEs, nearly all cargo molecules are sorted into recycling tubules or the ERC and returned to the PM, whereas very little enter LEs and lysosomes. *Type B cargo* travels along the bulk route that in EE/SEs, it directs some cargo into recycling tubules and some into LEs and lysosomes for degradation. *Type C cargo* is short-lived, travels along the degradative route, and nearly all cargo is sorted into LE and degraded in lysosomes. *Type D cargo* is long-lived, travels along the TGN retrograde route, and upon entry into EEs, it is sorted either into retrograde tubules to TGN or LEs and delivered to the TGN. *Type E cargo* is long-lived, travels

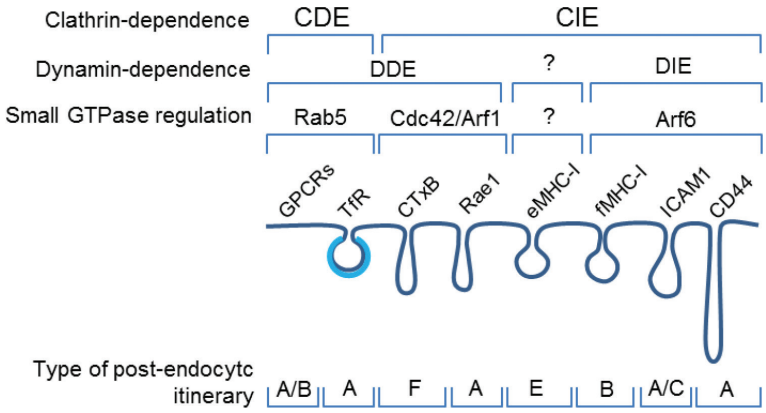



**Figure 1.** Typology of peripheral membrane cargo molecules used for kinetic analysis. Clathrin dependence during endocytic uptake classifies peripheral membrane cargo for clathrin-dependent endocytosis (CDE) and clathrin-independent endocytosis (CIE). The requirement of dynamin activity during pinching off endocytic carriers classifies cargo molecules as dynamin-dependent (DDE) and dynamin-independent (DIE). Cargo molecules can be classified into at least seven types according to the post-endocytic itinerary (see description in the text).

along the LE route, and in EE/SEs is sorted into LEs and recycled back to the PM either by recycling or exocytic carriers. *Type F cargo* travels along the Golgi retrograde route and from EE/SEs, it is sorted in the ERC from which it is delivered to the Golgi.

According to the classification, GPCRs can be classified as *Type A* and *Type B* [28, 38, 41] cargo, ligand-bound or antibody bound TfR as *Type A* cargo [9, 43], CTxB/GM1 as *Type F* [44]; Rae1 as *Type A* cargo [5, 6], eMHC-I as *Type E* cargo [13, 48], fMHC-I as *Type B* cargo [23, 43, 46, 48], ICAM1 as *Type A* and *Type C* cargo [47], and CD44 as Type A cargo [43, 46].

### 5.2. Cell surface expression displays internalization rate as integration of endocytic uptake and recycling of membrane proteins

Quantification of distribution of peripheral membrane proteins at the PM and in the endosomal system is critical for analysis and understanding their intracellular itineraries. Unfortunately, the number of techniques for accurate measurement of the number of peripheral membrane proteins in an intracellular compartment is quite limited. On the other hand, techniques based on antibody reagents and radioactively or fluorescently labeled ligands provide quite an accurate method for quantification of the number of peripheral membrane proteins at the cell surface. Thus, kinetic studies of cell surface expression of peripheral membrane proteins are the best available data for quantitative estimation of the intracellular itinerary, as explained below.

The steady state distribution of membrane endocytic cargo components reflects the net effect of endosomal sorting events. The frequency of appearance at the PM and endosomal compartment depends on the rate of transit between compartments and a compartment with the lowest rate of the transit will be a major retention site for cell surface receptor. In general, the distribution of peripheral membrane proteins will depend on the rate of endocytic uptake from the PM and the integrated rate of recycling from endosomal compartments. Receptors with higher endocytic rate will be retained inside the cell and receptors with higher recycling rate will be retained at the cell surface. For example, after rapid endocytic uptake, the TfR almost entirely enters into the recycling circuit, return to the cell surface, and again into endocytic carries. Very little (less than 1%) of internalized TfR is rerouted into LEs and degraded. Thus, TfR circulates from PM through endosomes and back. After several cycles, TfRs established the steady state distribution at the PM and endosomal compartment, and the net result is a redistribution of two-thirds of TfR inside the cell. On the contrary, the rate of recycling of MHC-I proteins overrides the rate of endocytosis, and thus the majority of MHC-I proteins reside at the cell surface. Inhibition of recycling or enhancement of endocytosis, thus, is a mechanism available to the cell to regulate cell surface level of a peripheral membrane protein.

### 5.3. Kinetic modeling of peripheral membrane protein trafficking

Kinetic models have been used to translate the cell physiology knowledge into mathematical formulas. Accordingly, the knowledge on endosomal compartments determined the number of compartments taken into analysis. Most of the kinetic studies were based on the use of a minimal number of compartments, and the recent expansion on the knowledge about EE system enabled the development of more complex models. The three models can be identified in the literature (**Figure 2**).

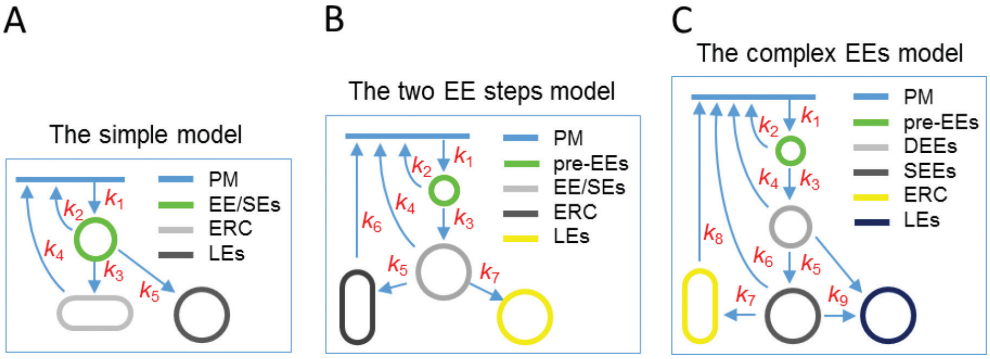



**Figure 2.** Kinetic models of peripheral membrane protein trafficking. (A) The simple model. (B) The two EE steps model—pre-EEs and EEs as sorting endosome. (C) The complex EEs model—pre-EEs and dynamic and static EEs. PM, plasma membrane; EE/SEs, early/sorting endosomes; ERC, endosomal recycling compartment; LEs, late endosomes; DEEs, dynamic early endosomes; and SEEs, static early endosomes.

The simple model (**Figure 2A**) used EE/SEs, the ERC, and LEs as the only recognized compartments of cargo transit [17]. With the expansion of the knowledge about the endosomal system, it is becoming clear that EEs, LEs, and even the ERC represent a various set of organelles and subcompartments and that the initial categorization into early, late, and recycling endosomes is oversimplified [10, 31, 35]. Recent studies introduced pre-EEs as a stable sorting station that sorts and recycles endocytotic cargo [25–28]. Thus, the EE system can be subdivided into at least two stages: proximal comprised of pre-EEs and the distal comprised of EE/SEs (**Figure 2B**). The more complex organization of the proximal stage (**Figure 3**), for example, has been proposed recently [26]. The funnel-like structure of EE system [8] suggests more complexity and requires separation of the EE system into at least two compartments (**Figure 2C**). Although the degree of biochemical and morphological separation of EEs is still incomplete, live-cell imaging studies [31, 32] suggest that EEs are organized into the more dynamic and static populations (**Figure 2C**).

To analyze endocytic itinerary of the seven peripheral membrane cargo molecules (**Figure 1**), we used the two-step model (**Figure 2B**). A kinetic model was developed which predicts sequential trafficking of peripheral membrane cargo molecules from PM through the endosomal system

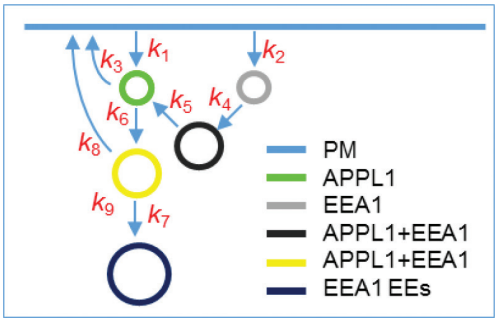



**Figure 3.** Pre-early endosome model proposed by Kalaidzidis et al. [26].

and that recycling may occur from every step of endosomal trafficking (pre-EEs, EEs/SEs, the ERC, and LEs). Our modeling was based on the following assumptions: (i) each compartment is considered homogenous, "well mixed," and can grow and shrink in size; (ii) cargo enters and leaves the compartments all the time; (iii) the cargo flux is presented as the fraction of cargo in the compartment at any given time; (iv) the velocities can change in space and time, continuously and smoothly; (v) the model does not capture the stochastic, "back and forth" movements of endosomes; (vi) the rates of exit from the compartment should not exceed the rate of entry into the compartment; (vii) different rates of exit from a compartment assume existence of different domains within a compartment; (viii) the predictions must be consistent with values derived from experimental data, including morphological analysis. The model predicts constant flux of cargo from a compartment toward the next compartment with the first-order rate kinetics [16, 17]. We predicted only the forward flux, and the retrograde flux, if exists, was integrated as a sum within the forward flux. Initially, the amount of cargo at the PM was set to 100(%), and the PM was considered as the first compartment. The assigned rate constants are graphically displayed in **Figure 2B**.

We fit the parameters of the model to the kinetic data of cargo molecule determined experimentally by flow cytometry, as cell surface expression of mAb-labeled or ligand-labeled peripheral membrane cargo molecules. To quantify the goodness of any of the models obtained by the described fitting processes, we used the coefficient of determination ($R^2$). The fitting was based on the adjustment of kinetic parameters until the $R^2$ value was larger than 0.93.

### 5.4. Determination of the endocytic rate constant

The endocytic rate constant is defined as the probability of a cell surface receptor to be internalized in 1 min at 37°C [49]. Thus, for kinetic analysis of the earliest post-endocytic events, it was critical to determine the endocytic rate constant ($k_1$). For experimental measurement of $k_1$ for peripheral membrane cargo molecules, it was essential to satisfy the following three conditions [49]: (i) internalized and cell surface cargo molecules can be quantitatively discriminated from each other, (ii) there is no degradation at the time of measurement, and (iii) there is no or very little dissociation of primary and secondary reagents during the course of the measurement. Thus, for each cargo molecules, we precisely defined the first step, conditions of ligand binding at the cell surface, since the pool of occupied cell surface molecules is the substrate for endocytosis. All primary and secondary reagents were tested for concentration and time required for saturation of almost all (>98%) PM molecules at 4°C, and dissociation rate of bound primary and secondary reagents at 4°C and at 37°C in the absence of endocytosis.

Experimental identification of $k_1$ was performed by fitting the predicted curve to the experimental data sets by iterative adjustment of $k_1$ values and visual alignment by minimization of the sum of square differences ($R^2$) [13, 42].

### 5.5. Selection of kinetic parameters

Kinetic parameters, rate constants ($k_1$-$k_8$) and the time of the beginning of transition between compartments, were manually chosen by fitting the PM level to the experimental data using the existing knowledge from the literature about endosomal kinetics.

Kinetic parameters for the pre-EE step were based on the following: (i) endocytic vesicles were observed after as little as 20 s of internalization [21]; (ii) after 2 min TfR localize in pre-EEs [25–27]; (iii) in the first 2 min, TfR does not localize in EEA1-positive endosomes, and partial colocalization with EEA1 is evident between 2 and 3 min; (iv) internalized cargo (TfR, EGFR) localize in EEs 3–6 min after endocytosis [17, 25, 27] and EEs are maximally filled with internalized cargo after 5 min [17]; (v) after 5 min, less than 10% of internalized TfRs localize in pre-EEs [27]. The recycling rate constant from pre-EEs ($k_2$) of 0.17–0.35 $min^{-1}$ was reported for EGFR and of 0.35–0.69 $min^{-1}$ for TfR [15]. The pre-EE-to-EE transition rate constant ($k_3$) of 0.15–0.35 $min^{-1}$ was reported for TfR [17, 18], 0.69 $min^{-1}$ for HDL, and 0.53 $min^{-1}$ for Glut4 [19].

Kinetic parameters for the EE step were based on the observation that the EE stage of endosomal trafficking ends 6–10 min after endocytosis [25, 37]. The EE recycling rate constant ($k_4$) of 0.11–0.35 $min^{-1}$ was reported for TfR and B2AR [10, 17, 18, 38]. Several reports suggest that the transition of cargo from SE to the ERC ($k_5$) occurs with the rate of approx. 0.30–0.35 $min^{-1}$ [37, 39]. The transport from SEs into LEs was reported to occur at the rate of 0.087–0.115 $min^{-1}$ [10, 15, 24, 37].

Kinetic parameters for the ERC step were based on the observation that the ERC is maximally filled at 12–13 min [17]. The ERC recycling rate constant ($k_6$) was determined in many studies based on loading of Tf-labeled TfRs. Most often reported rate was 0.040–0.080 $min^{-1}$ [10, 15, 17, 39], although lower and higher rates up to 0.3 $min^{-1}$ [19, 39] were reported. Similar externalization rate from the ERC was also reported using the fluorescent lipid analog C6-NBD-SM [39]. Recycling rate from LEs ($k_8$) of 0.058 $min^{-1}$ was reported for open MHC-I conformers [13].

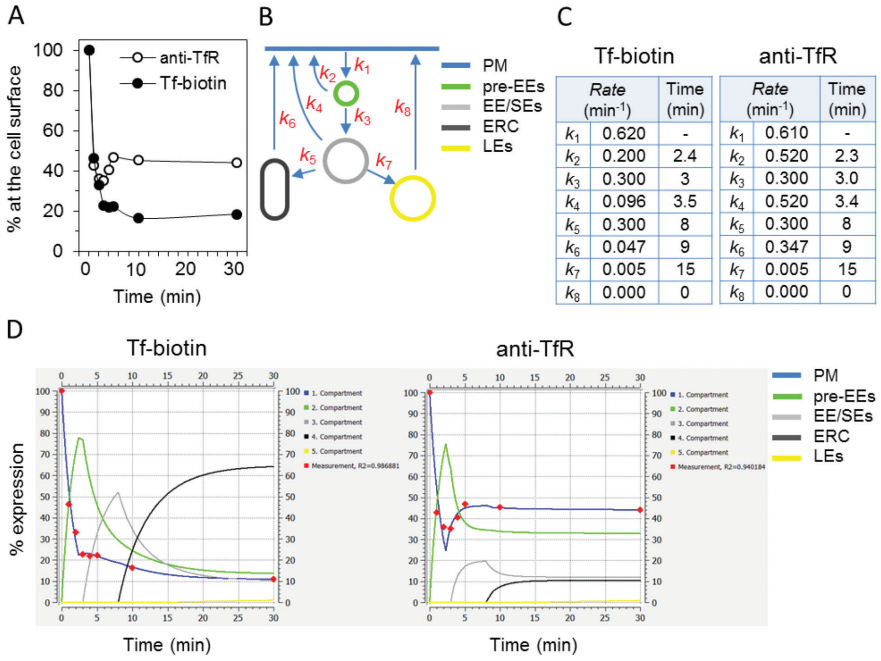
### 5.6. Evaluation of the kinetic model by identification of the rapid recycling of the transferrin receptor (TfR)

To evaluate our kinetic model, we analyzed the endocytic itinerary of TfR which is the best characterized CDE molecule in the literature. For quantification, we used its ligand transferrin (Tf) conjugated with the biotin (Tf-biotin) and specific monoclonal antibody (anti-TfR Abs). We used these two reagents since they provide different information about TfRs. Tf-biotin displays internalized receptors at the cell surface that are recycled back to the cell surface from a non-acidic but not from an acidic endosomal compartment. In contrast, anti-TfR Abs display all receptors that recycle back to the cell surface. TfRs were labeled with these two reagents at 4°C, cells were rapidly warmed to 37°C (internalization), and analyzed every minute for the number of labeled TfRs that remained at the cell surface (**Figure 4**).

Rapid loss of Tf-biotin- and anti-TfR-labeled TfRs from the cell surface indicates rapid endocytic uptake (endocytic rate, $k_1$) with the very high rate. With the estimated endocytic rate of ~0.61 $min^{-1}$, all receptors should be removed from the cell surface within 10 min. However, after 3 min and later, both Tf-biotin- and anti-TfR-labeled receptors remained at the cell surface (**Figure 4**), suggesting either the arrest of endocytosis or rapid return of labeled receptors back to the cell surface. In the presence of aluminum-fluoride, a potent inhibitor of endosomal recycling [11], labeled receptors did not remain at the cell surface (data not shown) suggesting that endocytosis normally goes on after 3 min and that the reason for the maintenance of the labeled receptors is rapid return by recycling.

To fit the predicted cell surface level to the experimental data, the rapid recycling from the pre-EEs ($k_2$) should be activated after 2.3–2.4 min after initiation of endocytosis (**Figure 4**).

http://dx.doi.org/10.5772/intechopen.75685




| Tf-biotin | Rate (min$^{-1}$) | Time (min) |
|---|---|---|
| $k_1$ | 0.620 | - |
| $k_2$ | 0.200 | 2.4 |
| $k_3$ | 0.300 | 3 |
| $k_4$ | 0.096 | 3.5 |
| $k_5$ | 0.300 | 8 |
| $k_6$ | 0.047 | 9 |
| $k_7$ | 0.005 | 15 |
| $k_8$ | 0.000 | 0 |

| anti-TfR | Rate (min$^{-1}$) | Time (min) |
|---|---|---|
| $k_1$ | 0.610 | - |
| $k_2$ | 0.520 | 2.3 |
| $k_3$ | 0.300 | 3.0 |
| $k_4$ | 0.520 | 3.4 |
| $k_5$ | 0.300 | 8 |
| $k_6$ | 0.347 | 9 |
| $k_7$ | 0.005 | 15 |
| $k_8$ | 0.000 | 0 |

**Figure 4.** Evaluation of the kinetic model by analysis of the post-endocytic itinerary of transferrin receptor. (**A**) *Internalization kinetics of TfRs.* Cell surface TfRs were labeled either with Tf-biotin or anti-TfR mAb at 4°C for 30 min, incubated at 37°C from one to 30 min, and stained with AF$^{488}$-conjugated secondary reagents. Cell surface fluorescence was quantified by flow cytometry. (**B**) *Schematic diagram of the kinetic model used for analysis of TfR post-endocytic itinerary.* (**C–D**) *Outcomes of the kinetic analysis*. Kinetic parameters are presented in table (**C**), and relative distribution within the endosomal system using these parameters is presented in the screen-shots of the kinetic modeling software for multicompartment analysis (**D**) [13, 42]. Experimental data (red diamonds) were plotted into the software, and rate constants ($k_1$–$k_8$) and time of the beginning transition between compartments (time) were adjusted to fit the curve of the predicted cell surface level (blue line) to the experimental data. Green, gray, dark gray, and yellow lines represent predicted distribution in corresponding endosomal compartments. The analysis was performed on the J26 fibroblast-like cell line.

Recycling from pre-EEs ($k_2$) should be active for the whole time of chase and in the absence of this mechanism, it was impossible to simulate the measured receptor levels at later times (data not shown). The estimated recycling rate from pre-EEs ($k_2$) was significantly lower for Tf-biotin- (~0.20 min$^{-1}$) than for mAb-labeled (~0.52 min$^{-1}$) TfRs (**Figure 4**), suggesting that a fraction of Tf-biotin/TfRs was returned to the PM from pre-EEs that are sufficiently acidic to convert *holo*-Tf into *apo*-Tf. Namely, a fraction of TfRs loaded with *apo*-Tf-biotin that was delivered from acidic endosomes would not be detected by flow cytometry as *apo*-Tf is released from the receptor that reached the cell surface. Nevertheless, the kinetic analysis demonstrates that endocytosed TfRs must be rapidly recycled to maintain the cell surface level. The rate of recycling must be rather high to oppose the high endocytic rate.

Entry of internalized TfRs into EEs occurred 3–4 min after endocytic uptake with the rate ($k_3$) similar to that described in the literature [17–19], followed by immediate activation of recycling from this compartment ($k_4$). Estimated recycling rate using Tf-biotin (**Figure 4**) represents the rate of TfR recycling sufficient to return *holo*-Tf-biotin-associated receptors that are detected by flow cytometry. Recycling of *holo*-Tf-biotin-associated receptors was also detected from the ERC ($k_6$).

Thus, Tf-biotin detection at the cell surface suggests that the rate of recycling of TfRs from EEs and the ERC is significantly higher than observed by measuring the *apo*-Tf release [10, 15, 17–19, 38, 39]. This observation is confirmed by estimations of the recycling rates from EEs and the ERC ($k_4$ and $k_6$) of mAb-labeled receptors (**Figure 4**). The omitting of any of these recycling rates made impossible to simulate measured cell surface levels of receptors. Thus, kinetic analysis of Tf-biotin- and mAb-labeled TfRs demonstrates the existence of the rapid recycling of TfRs from non-acidic endosomes and recycling with the high rate at the entire recycling circuit.

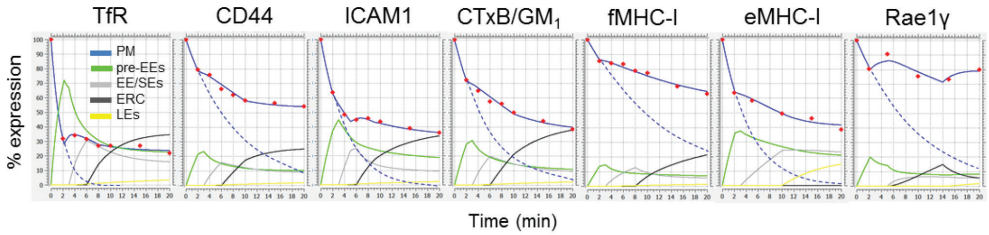
### 5.7. Kinetic modeling provides evidence for rapid recycling of clathrin-independent peripheral membrane proteins

We analyzed cell surface kinetics and intracellular itinerary of six CIE cargo molecules, five PM proteins using mAb reagents and $GM_1$ using biotinylated CTxB. The PM kinetics was measured using flow cytometry as described for TfR. The incubation conditions and the shift in temperature (i.e., from 4°C to 37°C and vice versa) were carefully controlled to minimize the time required for warming or cooling. Experimental details and validation of protocols have been published in several papers [13, 22, 23, 42, 48].

Experimentally acquired values were imported into the kinetic modeling software [13, 42], and kinetic analysis of trafficking through five membranous compartments (**Figure 4**) was performed. The fitting of kinetic parameters (rate constants and transition time) was based on data from the immunofluorescence analysis of intracellular itinerary and data from the literature. The experimental data and the outcome of the fitting procedure (including distribution throughout the compartments) for each cargo molecule are presented in graphs of **Figure 5**, and parameters that best fit to the experimental data in the table of **Figure 5**.

PM kinetics of six CIE cargo molecules, which are endocytosed by distinct mechanisms, demonstrated different rates of disappearance from the cell surface ($k_1$). CDE and DDE cargo, such as TfR, disappeared with the very high rate (0.573 $min^{-1}$) also in murine embryonal fibroblasts (**Figure 5**) and other cell lines used for analysis (data not shown). CIE and DDE cargo (such as $GM_1$/CTxB and ICAM1) disappeared with the moderate rate, whereas CIE and DIE cargo (CD44, fMHC-I, and Rae1) disappeared from the cell surface six times slower than TfR (**Figure 5**). Although the contribution of dynamin in the endocytic uptake of eMHC-I has not been proven, kinetic of their disappearance from the cell surface suggests that they belong to the group of CIE and DDE cargo and may use the similar route as ICAM1 and $GM_1$/CTxB (**Figure 5**).

The rate of disappearance from the cell surface represents the rate of endocytosis ($k_1$). The observed rates ($k_1$ for all six CIE cargo molecules) would lead to the kinetics of the loss (internalization) from the plasma membrane as presented by blue dashed lines in the graphs of **Figure 5**. Apparently, the measured kinetics of cell surface expression of all cargo molecules is distinct to this calculated kinetics, and all cargo molecules remain at the cell surface much longer than predicted by the calculated kinetics. As described above for TfR, the prolonged retention of cargo molecules at the cell surface is not due to the arrest in the endocytic uptake, since under conditions of inhibited recycling by aluminum-fluoride [11], all cargo molecules disappeared from the cell surface with the rate similar to that predicted by the calculated kinetics (data not shown). Thus, the reason for the prolonged maintenance of cargo molecules at the cell surface is the activation of the recycling mechanism, which returns internalized cargo to the cell surface.




| | TfR | | CD44 | | ICAM1 | | CTxB | | fMHC-I | | eMHC-I* | | Rae1** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $min^{-1}$ | time | $min^{-1}$ | time | $min^{-1}$ | time | $min^{-1}$ | time | $min^{-1}$ | time | $min^{-1}$ | time | $min^{-1}$ | time |
| $k_1$ | 0.573 | 0 | 0.120 | 0 | 0.231 | 0 | 0.165 | 0 | 0.073 | 0 | 0.210 | 0 | 0.101 | 0 |
| $k_2$ | 0.355 | 2.2 | 0.330 | 2 | 0.145 | 2 | 0.310 | 2 | 0.375 | 2 | 0.320 | 2.1 | 0.655 | 2.2 |
| $k_3$ | 0.250 | 3 | 0.310 | 3 | 0.310 | 3 | 0.310 | 3 | 0.310 | 3 | 0.110 | 3 | 0,310 | 5 |
| $k_4$ | 0.108 | 4 | 0.038 | 4 | 0.315 | 5 | 0.058 | 4 | 0.088 | 4 | 0 | 0 | 0.088 | 5 |
| $k_5$ | 0.250 | 6 | 0.310 | 6 | 0.310 | 6 | 0.310 | 6 | 0.310 | 8 | 0 | 0 | 0.310 | 10 |
| $k_6$ | 0.108 | 8 | 0.105 | 10 | 0.068 | 9 | 0.058 | 10 | 0.038 | 10 | 0 | 0 | 0.388 | 14 |
| $k_7$ | 0.010 | 15 | 0.010 | 15 | 0.010 | 20 | 0.010 | 15 | 0.010 | 15 | 0.110 | 10 | 0.060 | 15 |
| $k_8$ | - | - | - | - | - | - | - | - | - | - | 0.130 | 10 | - | - |

**Figure 5.** Analysis of the early post-endocytic itinerary of clathrin-dependent and clathrin-independent cargo molecules. PM proteins were labeled with specific mAbs and ganglioside $M_1$ ($GM_1$) with $AF^{488}$-CTxB at 4°C, and cell surface expression was quantified by flow cytometry after indicated time of internalization at 37°C. The experimental data (red diamonds) were loaded into the software for multicompartment analysis [13, 42], five endosomal compartments setup as described in **Figure 4**, and kinetic parameters were adjusted by fitting the curve of the predicted cell surface level (full blue line) to the experimental data. Kinetic rates ($k_1$–$k_8$) and the time of the beginning of transport between compartment (time) are shown in the table. Dashed blue lines represent predicted cell surface expression in the absence of recycling. The analysis was performed on murine embryonal fibroblasts except for eMHC-I (*), which was analyzed on $L^d$-transfected L-cells (L-$L^d$), and Rae1γ (**), which was analyzed on Rae1γ-transfected NIH 3 T3 cells.

Alignment of kinetic parameters to the experimental data between 2 and 5 min after initiation of internalization, demonstrated that recycling mechanism should be activated approx. within 2 min for all CIE cargo molecules to explain experimentally measured value at 5 min (**Figure 5**). Therefore, our analysis demonstrates that also CIE cargo, irrespective of the involvement of the dynamin in the endocytic uptake, enter into endosomes that may recycle cargo back to the cell surface very early after endocytosis. Apparently, the recycling mechanism is activated from pre-EEs, since at 2 min, none of the cargo molecules should be expected in EEs. The estimated rate of exit from pre-EEs indicates rather a high rate (more than 0.3 $min^{-1}$), which is in agreement with previously observed rapid recycling kinetic [15, 17–19]. The only exception was ICAM1 which activates very fast recycling mechanism later. The distinct rate of the return from pre-EEs indicates that pre-EEs are not the homogenous population of endosomes. This is not surprising since distinct endocytic routes lead to distinct endocytic carriers which have membrane composition that correspond to the piece of the PM taken up into endocytic carriers. Altogether, this analysis indicates that rapid recycling mechanism exists for all CIE cargo molecules and occurs very early after endocytosis with a very high rate.

Analysis of further steps (5 min after internalization) in the endocytic itinerary demonstrate that additional recycling should be activated, from EEs and later from the ERC, to maintain the measured cell surface level. The kinetics of recycling from these compartments ($k_4$ and $k_6$) differ among cargo molecules, indicating that EEs and the ERC may have distinct domains or subcompartments that generate recycling carriers with distinct rates. Although cargo molecules

used for this analysis differ in their post-EE endocytic itinerary, we did not add this complexity into the analysis since our intention in this discussion was to demonstrate that all endocytic cargo undergoes into the rapid recycling circuit. The rapid recycling circuit is, apparently, distinct from the conventional recycling circuit [1] and also recycles cargo that is excluded from the conventional recycling circuit. For example, although eMHC-I molecules are excluded from the recycling circuit that involves EEs and the ERC [48] and recycles from LEs [13], they are included onto the rapid recycling circuit and recycled from pre-EEs with the relatively high rate (**Figure 5**). Given that the conventional recycling circuit has the capacity of conformation-based sorting (i.e., sorting of membrane proteins that changed conformation and can be considered as misfolded), this type of endosomal sorting does not operate in the rapid recycling circuit.

Altogether, our analysis of post-endocytic itinerary of seven cargo molecules that utilize distinct endocytic machinery and undergo distinct post-endocytic itinerary demonstrated that all endocytic cargo molecules enter into the rapid recycling circuit and are rapidly returned to the cell surface after endocytosis. Thus, a critical segment of post-endocytic trafficking of peripheral membrane proteins and other membrane components was underestimated in the past and should be considered in the explanation of the cellular physiology of peripheral membrane proteins and related physiological and pathophysiological processes.

## 6. Physiological significance of the rapid endosomal recycling

Very early activated recycling (rapid recycling) is apparently an important cellular physiology mechanism to reduce energy consumption for the maintenance of plasma membrane composition. The vast majority of the energetic cost for the maintenance of the cells is associated with protein synthesis and maintenance of membranes (i.e., ~30% cellular energy budget is spent on membranes) [14]. Therefore, there is an evolutionary rationale to evolve mechanism(s) which will reduce energy consumption in the membrane trafficking pathways. For example, although the bulk recycling route may provide sufficient amount of a protein at the cell surface, for some cellular proteins (i.e., proteins that maintain cell–cell contacts), it is not reasonable to enter deep inside the cell and travel throughout the entire recycling circuit. It would be more energy efficient to return internalized proteins back to the cell surface as soon as possible. To maintain the steady-state distribution of a protein at the cell surface, which is determined by the post-endocytic itinerary, it is essential to synthesize enough proteins to fill all compartments that are on the route. Thus, the size of the cellular pool of a protein would depend on the length of a route intracellular trafficking of a protein. The rapid return would require less protein synthesis and would reduce a load of endosomal compartments by a protein, which may be important for fidelity of post-endocytic sorting events. Therefore, rapid recycling may reduce energy consumption by the shortening of the recycling circuit for many membrane components and by reducing the number of membrane proteins required to fill the membranous system of the cell properly.

Rapid recycling is also essential for the cellular physiology of cholesterol homeostasis, which requires efficient binding of lipoproteins (LDL and VLDL remnants) to LDL receptor (LDLR), their internalization, the release of lipoproteins in the endocytic compartment, and the return of receptors to the cell surface for further rounds of lipoprotein uptake. Quantitative fluorescence imaging study [50] demonstrated that lipoprotein release occurs before the entry of LDLRs

into EEs. The failure of LDLRs recycling results in the loss of receptors by rerouting into the lysosomal degradation pathway and several therapeutic strategies were explored in order to minimize LDLRs degradation. The failure of pre-EE recycling route also may result in reduced LDL clearance. Thus, understanding the rapid LDLRs' recycling pathway may lead to the identification of new therapeutic targets that can be exploited to prolong LDLR half-life and, thereby, enable treatment of atherosclerosis-based diseases, including coronary artery disease.

Very early activation of recycling is undoubtedly vital in shaping cellular receptor-mediated signaling via GPCRs. Rapid endocytic recycling determines the number of functional receptors at the PM [28, 38] and the rapid recovery of functional signaling after ligand-activated endocytic uptake [28, 38]. Even more, a rapid recycling process that delivers receptors at high concentration may be particularly suited for dynamic regulation of localized receptor signaling.Rapid recycling may be essential for assembly and maintenance of cell-cell contacts. The very fast rate of endocytic uptake may result in the redistribution of molecules that maintain cell contact, and slow recycling processes may make cell contacts weaker. It has been shown that recycling processes are important for regulation of trafficking of various cell adhesion molecules, including cadherins and integrin receptors (reviewed in [51]). Alteration of recycling processes has been shown to be associated with the loss of cell adhesion increased motility and cell migration, which are the characteristics of epithelial-mesenchymal transition and invasive cancer cells [51]. Many of these processes may be assigned to the rapid recycling. Thus, misregulation of the rapid recycling can result in human disease when it compromises important cellular functions, such as lipid homeostasis, cellular signaling, movement, or division.

## 7. Conclusion(s)

Although significant progress has been made in understanding the endosomal recycling, characterization of endosomal recycling routes of peripheral membrane proteins is still poorly integrated into the cellular physiology, especially into the higher-order physiology [11]. The increasing number of molecules with characterized recycling routes indicates that recycling may occur very early in the endocytic tract, suggesting more complexity of the endosomal recycling circuit and the need for its integration into physiology and pathophysiology of many cellular processes.

Our study demonstrates that the constitutive endocytic uptake of peripheral membrane proteins occurs with much higher rate and the overall low internalization rate is primarily maintained by rapid recycling prior their entry into structured EE network. Internalization rates and endocytic rates described in the literature, in fact, represent the combination of endocytic uptake and the (rapid) recycling processes. Rapid recycling, therefore, should be taken into consideration when analyzing and estimating many important cellular processes, including physiology cell motility and adhesion, receptor signaling, lipoprotein metabolism, and signal transduction.

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## Conflict of interest

The authors declare that they have no conflict of interest.

## Notes/Thanks/Other declarations

We apologize to all authors whose work has not been cited owing to space restrictions, and for not always citing primary literature.

## Abbreviations

| | |
|---|---|
| Ab | antibody |
| AF | alexa-fluor |
| CDE | clathrin-dependent endocytosis |
| CIE | clathrin-independent endocytosis |
| CTxB | cholera-toxin B subunit |
| DDE | dynamin-dependent endocytosis |
| DIE | dynamin-independent endocytosis |
| EE | early endosome |
| ERC | the endosomal recycling compartment |
| GPCR | G protein-coupled receptor |
| GTPase | guanosine-triphosphatase |
| LE | late endosome |
| mAb | monoclonal antibody |
| MHC-I | major histocompatibility class I |
| PM | plasma membrane |
| pre-EE | pre-early endosome |
| SE | sorting endosome |
| Tf | transferrin |
| TfR | transferrin receptor |
| TGN | the *trans*-Golgi network |

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