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Citation for the original published paper (version of record):

Voss, S., Li, F., Raetz, A., Roeger, M., Wu, Y-W. (2019)
Spatial Cycling of Rab GTPase, Driven by the GTPase Cycle, Controls Rab's Subcellular
Distribution
Biochemistry, 58(4): 276-285
<https://doi.org/10.1021/acs.biochem.8b00932>

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

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<https://pubs.acs.org/doi/10.1021/acs.biochem.8b00932>

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Spatial cycling of Rab GTPase, driven by GTPase cycle, controls Rab's subcellular distribution

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Running title:

Spatial cycling of Rab controls Rab subcellular distribution

Abstract

Rab GTPases (> 60 members in human) function as master regulators of intracellular membrane trafficking. Correct and specific localization of Rab proteins is required for their function. It remains elusive how the distinct spatial distribution of Rab GTPases in the cell is regulated. To make a global assessment on the subcellular localization of Rab1, we determined kinetic parameters of two pathways that control the spatial cycles of Rab1, i.e. vesicular transport and GDP dissociation inhibitor (GDI)-mediated recycling. We demonstrate that the switching between GTP- and GDP-binding states, which is governed by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), GDI and GDI displacement factor (GDF), is a major determinant for Rab1's ability to effectively cycle between cellular compartments and eventually for its subcellular distribution. *In silico* perturbations of vesicular transport, GEFs, GAPs, GDI and GDF using a mathematical model with simplified cellular geometries showed that these regulators play an important role in subcellular distribution and activity of Rab1.

Keywords: Rab GTPase, Rab1, membrane trafficking, membrane targeting, dynamics, GDP dissociation inhibitor (GDI), GDI displacement factor (GDF), guanine nucleotide exchange factor (GEF), GTPase activation proteins (GAP), mathematical simulation.

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Introduction

Rab proteins are the key regulators of vesicular trafficking in cells. Through their interaction with different effectors, Rabs are involved in all steps of intracellular vesicular transport ranging from cargo selection, vesicle budding and uncoating, vesicle transport to vesicular fusion.¹ Each Rab features a specific subcellular localization and a subset of interacting partners that govern their particular function.²

Rabs belong to the family of small GTPases that exert their regulatory role by acting as molecular switches. They cycle between an active GTP-bound and an inactive GDP-bound form, which is termed “GTPase cycle”. The nucleotide binding state is tightly regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs catalyze the exchange of GTP for bound GDP, while GAPs accelerate the slow intrinsic GTP hydrolysis of GTPases.³ In their active, GTP-bound form, these proteins interact with effector proteins that initiate downstream signaling.

Rab membrane attachment is mediated through C-terminal lipophilic geranylgeranyl groups. Cycling between the cytosol and membranes is an essential feature of the mode of action of Rabs and is made possible by reversible interaction with GDP dissociation inhibitor (GDI). GDI solubilizes and extracts prenylated Rab GTPases from membranes by binding and shielding the hydrophobic geranylgeranyl groups.⁴⁻⁷ Membrane-bound GDI displacement factors (GDFs) were proposed to disrupt GDI:Rab complexes, leading to insertion of the prenylated Rab into the membrane in the GDP form and release of GDI into the cytosol.^{8,9} So far only one GDF protein, prenylated Rab acceptor protein 1 (PRA1)/Yip3 (the yeast homolog of PRA1), has been identified.^{10,11}

Rab1 plays a key role in endoplasmic reticulum (ER)-to-Golgi transport, maintenance of Golgi structure and autophagosome biogenesis during autophagy.¹²⁻¹⁷ Rab1 is hijacked, activated and post-translationally modified by *Legionella* effector proteins when the bacteria invade host cells.¹⁸⁻²³ Rab1 is activated by a large multisubunit complex, transport protein particle (TRAPP) complex, which also functions in tethering.²⁴⁻²⁶ TBC1D20 has been identified as a Rab1 specific GAP.^{13,27} In accordance with its role in ER-to-Golgi vesicular transport, Rab1 localizes to the ER and Golgi as well as their intermediary compartments.¹²

Despite their well-documented roles in cellular trafficking, it is still not fully understood how the distinct spatial distribution of Rab GTPases in the cell is regulated.^{1,9,28-32} To explore the mechanisms of Rab membrane targeting, we examined the kinetic parameters of Rab1 spatial cycling under different conditions. We found that Rab switching between different nucleotide binding states is the driving force for their spatial cycles between cellular compartments. We further substantiate our experimental findings by fitting experimental data to a mathematical model with simplified cellular geometries, which integrates the measurements from different experimental approaches into a comprehensive picture of Rab1 spatial cycling. The *in silico* model is used to evaluate the role of

different regulators in subcellular distribution and activity of Rab1 in the cell. Taken together, the spatial cycles of Rab1 determine its subcellular distribution. The spatial cycling is coordinated by the GTPase cycle that is regulated by GEFs, GAPs, GDF and GDI.

Experimental Details

Cell culture and transient transfection

HeLa cells were cultured in 100 mm tissue culture dishes (Sarstedt) and 7-8 ml minimum essential medium (MEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 1 % (v/v) sodium pyruvate solution (100 mM), 1 % GlutaMAX and 1 % (v/v) non-essential amino acids (all Thermo Fisher Scientific) at 37 °C and 5 % CO₂. Cells were split every two to three days at a confluency of 70-80 %. For transient transfection 0.5-1 ml cell suspension was added to a mixture of 1-3 µg plasmid DNA preincubated for 15-20 min in 200 µl Opti-MEM (Thermo Fisher Scientific) with 1-2 µl X-tremeGENE HP transfection reagent in a 35 mm glass bottom dish (MatTek). After transfection the cells were incubated overnight at 37 °C and 5 % CO₂ and subsequently used for live cell microscopy.

PRA1 and TRAPPC4 knock-down cell lines

For PRA1 and TRAPPC4 (TRAPP complex subunit 4, mammalian homolog of yeast Trs23) shRNA constructs, we use pLL3.7 vector (Addgene #11795), which encodes puromycin for selection in mammalian cells. After 2 weeks selection in the presence of 1 µM puromycin, the knock-down efficiency was confirmed by western blot (**Fig. S17, S18**) with anti-PRA1 antibody (EPR1747Y, Novus Biologicals) and anti-TRAPPC4 antibody (PA5-44630, Thermo Fisher).

Nocodazole treatment

To disrupt vesicular transport, cells were incubated on ice for 30 min in imaging medium with 5 µg/ml nocodazole and then were warmed to 37 °C prior to live cell imaging.

Microscopy and imaging

The FRAP and FLAP experiments were carried out using a TCS SP5 microscope (Leica) equipped with a 63x/1.4 HCX PL APO (λ blue) oil immersion objective (Leica). Lasers and settings used for fluorophore excitation, bleaching, photoactivation and the respective detection filter settings are listed below. All measurements were carried out in an incubation chamber at 37 °C and 5 % CO₂.

Excitation of	Laser	λ_{ex} (nm)	Emission filter settings (nm)
EGFP	Argon Laser LGK 7872 ML05	488	505-555
mKate2	561 DPSS YLK 6120 T02	561	595-685
Bleaching of EGFP	Argon Laser LGK 7872 ML05	488	-

Photoactivation of paGFP	Cube 1162002/AF	405	-
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The general setup of a FRAP experiment includes: (1) pre-bleaching imaging, (2) bleaching of the designated regions of interest (ROI) and (3) post-bleaching imaging to monitor the fluorescence recovery in the bleached area. At least three images were collected before bleaching, followed by extensive illumination (10 repetitions over 12 s) with maximal laser power at 488 nm (100 %, Argon Laser LGK 7872 ML05). After bleaching, the fluorescence recovery was monitored over a period of at least 400 s with images collected every 2-10 s.

A FLAP experiment consists of three steps: (1) image acquisition before photoactivation, (2) photoactivation of paGFP at the predefined ROI and (3) imaging of the redistribution of the protein with fluorescence. In the pre-activation step for each experiment, three images were collected. Photoactivation of paGFP-Rab1 was performed through illumination at 405 nm with 30-50 % of the maximal laser intensity (Cube 1162002/AF). Post activation images of paGFP-Rab1 and mKate2-giantin were collected every 2 s over a period of at least 300 s.

FRAP and FLAP analysis

The mean fluorescence intensity at the ROI was determined using the FRAP profiler plugin of the ImageJ Software and normalized against the fluorescence intensity of the whole cell. The normalized fluorescence was further normalized using Eq 1:

$$I_{\text{norm}}(t) = \frac{I(t) - I_0}{I_{\text{pre}} - I_0} \quad \text{Eq. 1}$$

Here $I(t)$ is the normalized fluorescence intensity at time t , I_0 the initial residual fluorescence after bleaching and I_{pre} is the mean intensity before photobleaching. The recovery of the mean fluorescence in the ROI was fitted to a single exponential function Eq. 2.

$$I_{\text{norm}}(t) = I_{\infty} (1 - e^{-k_{\text{obs}} \cdot t}) \quad \text{Eq. 2}$$

$$t_{1/2} = \frac{\ln(2)}{k_{\text{obs}}} \quad \text{Eq. 3}$$

I_{norm} corresponds to the fluorescence intensity at time t , I_{∞} is the fluorescence intensity after completed recovery and k_{obs} (k_{on}) is the observed rate constant for fluorescence recovery. The half-time of the fluorescence recovery ($t_{1/2}$) was derived using Eq. 3.

For photoactivation of paGFP-Rab1, the ROI was identified using mKate2-giantin as a Golgi marker. The mean fluorescence paGFP-Rab1 intensity at the ROI was quantified as the ratio of paGFP to mKate2-giantin and normalized as described above (Eq. 1).

$$I(t) = \frac{I(t)_{\text{paEGFP}}}{I(t)_{\text{mKate}}} \quad \text{Eq. 4}$$

To determine k_{on} for the Rab1 trafficking from the cytoplasm to the Golgi, the observed fluorescence increase at the Golgi following photoactivation at the cytoplasm was fitted to a single exponential function (Eq. 2).

For the reverse process of Rab1 trafficking from the Golgi to the cytoplasm, the fluorescence depletion from the Golgi after photoactivation was monitored. The resulting trace was fitted to a single exponential function (Eq. 5) to yield k_{off} .

$$I_{norm}(t) = I_{\infty} + A \cdot e^{-k_{obs} \cdot t} \quad \text{Eq. 5}$$

***In cellulo* prenylation assay**

The assay was performed as previously reported.³³ HeLa cells expressing GFP-Rab1b were washed three times with ice-cold PBS. Cells were lysed in 150ul lysis/prenylation buffer (25 mM HEPES, pH 7.2, 50 mM NaCl, 2 mM MgCl₂, 2 mM DTE, 20 μM GDP, 0.5% NP-40, 1x complete mini EDTA-free protease inhibitors (Roche, cat. no. 11836170001)). 50 μl of freshly prepared lysate (~1 μg/μl) were added with 2 μM RabGGTase, 2 μM REP-1 and 5 μM Biotin-GPP to initiate the prenylation reaction and incubate for 4 hr at room temperature. The reaction was quenched by adding 10 μl of 6× SDS sample buffer. The samples were boiled for 5 min at 95°C. The biotinylated Rab1 (unprenylated Rab1) was detected with primary anti-biotin (D5A7, Cell Signalling) and anti-GFP(AS-29779, AnaSpec) antibodies by western blotting.

Mathematical simulation

See Supporting Information.

UniProt Accession ID for proteins: Rab1b (Q9H0U4), PRA1 (Q9UI14), TRAPPC4 (Q9Y296)

Results

Subcellular localization of Rab1 is highly dynamic

To assess the dynamics of Rab1 trafficking between the cytoplasm and the Golgi apparatus, we performed fluorescence recovery after photobleaching (FRAP) and fluorescence localization after photoactivation (FLAP) experiments (**Fig. 1**).^{34, 35} Rab1 trafficking from the cytoplasm to the Golgi region was monitored by recovery after photobleaching of enhanced green fluorescent protein (EGFP)-Rab1 at the Golgi or by selective photoactivation of photoactivatable GFP (paGFP)-Rab1 in the cytoplasm. The opposite direction of Rab1 trafficking from the Golgi to the cytoplasm was monitored by photoactivation of paGFP-Rab1 at the Golgi region.

Due to the fluidity of the Golgi membrane,³⁶ it is crucial that photobleaching covers the entire Golgi region to exclude the recovery through lateral diffusion of unbleached Golgi-localized EGFP-Rab1. Contribution to the recovery from newly synthesized protein was excluded through treatment with the protein synthesis inhibitor cycloheximide (**Fig. S10**). Photobleaching over the time course of time-lapse imaging was determined to be less than 5 % of the initial fluorescence intensity (**Fig. S11**).

FRAP experiments for EGFP-Rab1 delivery from the cytoplasm to the Golgi compartment led to a rate constant (k_{on}) of $(1.6 \pm 0.4) \times 10^{-2} \text{ s}^{-1}$ (**Fig. 1 A and C, Fig. S12 A, Movie S1**). The corresponding recovery half time is $t_{1/2} = 43 \text{ s}$. The rate constant for Rab1 delivery to the Golgi was further confirmed by a FLAP experiment (**Fig. 1 B and D, Fig. S12 B, Movie S2**). Following photoactivation of the cytoplasmic paGFP-Rab1 pool, the increase in Rab1 fluorescence on the Golgi membrane was quantified to obtain the apparent rate constant k_{on} of $(1.9 \pm 0.4) \times 10^{-2} \text{ s}^{-1}$ ($t_{1/2} = 36 \text{ s}$) following photoactivation of wild type paGFP-Rab1. After photoactivation, multiple paGFP-Rab1 positive vesicular structures throughout the cell were observed trafficking towards the Golgi apparatus (**Movie S2**).

FLAP experiment for paGFP-Rab1 trafficking from the Golgi to the cytoplasm led to a rate constant (k_{off}) of $(0.3 \pm 0.1) \times 10^{-2} \text{ s}^{-1}$ ($t_{1/2} = 231 \text{ s}$) (**Fig. 2, Movie S3**), which is significantly slower than Rab1 delivery to the Golgi ($k_{on} = 1.6\text{-}1.9 \times 10^{-2} \text{ s}^{-1}$). The difference between the Rab1 influx and outflux to the Golgi leads to the enrichment of Rab1 molecules at the Golgi apparatus at the steady state (**Fig. 4 A and G**). Collectively, these findings underscore the highly dynamic nature of Rab1 trafficking, with a continuous flux of Rab1 molecules between the Golgi and the cytoplasm.

GTPase cycle is required for Rab1 spatial cycling

The function of Rab1 mutants that harbor functional deficiencies, rendering them either constitutively active (Q67L) or inactive (S22N or N121), have been studied previously.^{14, 37, 38} Transient expression of dominant inactive Rab1 mutants (S22N or N121) leads to disruption of the Golgi complex and relocation of Golgi and ERGIC resident proteins to peripheral structures. The effect of Rab1Q67L expression is less severe, as the overall Golgi structure remains intact.¹⁴ This is likely due to Rab1Q67L's ability to interact with effector proteins that mediate ER-to-Golgi transport. Barr and coworkers demonstrated that Rab1 activity is critical for the biogenesis and maintenance of functional Golgi structure¹³. When Rab1 activation is inhibited, e.g. through GAP overexpression, expression of a dominant-negative Rab1 mutant or Rab1 depletion through siRNA, anterograde ER-to-Golgi transport is blocked and the Golgi complex disintegrates.^{13, 14}

We sought to assess the effect of the GTPase deficient mutations on the dynamics of Rab1 spatial cycling. FRAP experiments of EGFP-Rab1Q67L showed that the rate for Rab1 delivery to the Golgi is significantly slower than that of the wild type protein, $k_{on}(Q67L) = (1.1 \pm 0.4) \times 10^{-2} s^{-1}$ and $k_{on}(wt) = (1.6 \pm 0.4) \times 10^{-2} s^{-1}$, respectively (**Fig. 3 A, Fig. S13 A and B, Movie S4**). FLAP experiments of paGFP-Rab1Q67L led to a similar observation, $k_{on}(Q67L) = (1.0 \pm 0.4) \times 10^{-2} s^{-1}$ and $k_{on}(wt) = (1.9 \pm 0.4) \times 10^{-2} s^{-1}$, respectively (**Fig. 3C, Fig. S13 C, Movie S5**). Furthermore, large Rab1-positive vesicles enriched in the cytoplasm and the relative Rab1 fluorescence intensity at the Golgi versus the cytoplasm was significantly reduced in cells expressing Rab1Q67L in comparison to cells expressing Rab1 wild type (**Fig. 4 B and H**). These results suggest that the enzymatic GTPase cycle is required for Rab1 spatial cycling and subcellular distribution.

The TRAPP II complex has GEF activity towards Rab1 with four conserved subunits (Trs23, Bet5, Trs31 and Bet3) being essential for GEF activity. Mutations in Trs23 (its mammalian homolog TRAPP complex subunit 4, TRAPPC4) abrogate GEF activity.^{39, 40} Knockdown of TRAPPC4 in cells led to fragmentation of the Golgi complex and accumulation of Rab1 and RabQ67L in the cytoplasm (**Fig. 4C, D, H, and Fig. S14**). These suggest that GEF-mediated nucleotide exchange is required for Rab1 subcellular distribution.

Another important regulator involved in the GTPase cycle is GDI, which prevents Rab from activation and mediates Rab recycling from membranes. As the affinity of GDI towards Rab proteins is strictly dependent on the nucleotide-binding state with preference for GDP-bound Rab, GDI-mediated recycling should be hampered for the constitutively active Rab1Q67L.⁹ Interestingly, the rate for Rab1Q67L trafficking from the Golgi to the cytoplasm $k_{off}(Q67L) = (0.6 \pm 0.1) \times 10^{-2} s^{-1}$ is significantly higher than that of Rab1 wild type $k_{off}(wt) = (0.27 \pm 0.1) \times 10^{-2} s^{-1}$ (**Fig. 3 B and D, Fig. S13 D, Movie S6**). This can be attributed to Rab1Q67L's higher percentage of (faster) vesicular transport from the Golgi

and reduction of (slower) GDI-mediated recycling (see next section). Accordingly, the relatively higher k_{off} also leads to less accumulation of Rab1Q67L at the Golgi (**Fig. 4 B, and H**).

GDI-mediated non-vesicular transport is required for Rab1 spatial cycling

To assess the role of non-vesicular transport in Rab1 cycling, we treated Rab1-transfected cells with nocodazole and quantified Rab1 trafficking by FRAP and FLAP experiments. Nocodazole is a tubulin-binding agent that interferes with microtubule polymerization and thus abrogates microtubule dependent vesicular transport. Nocodazole treatment leads to fragmentation of the Golgi complex and formation of ministacks at the ER exit sites, due to disruption of ER-to-Golgi transport (**Fig. 4 G and I**).⁴¹ Upon nocodazole treatment, the rate for Rab1 delivery to the Golgi was reduced by approximately 40% with $k_{\text{on}}(\text{FRAP}) = (0.9 \pm 0.3) \times 10^{-2} \text{ s}^{-1}$ and $k_{\text{on}}(\text{FLAP}) = (1.2 \pm 0.3) \times 10^{-2} \text{ s}^{-1}$ (**Fig. 3 A, Fig. S15 A-D, Movie S7, S8**). This indicates that vesicular transport plays an important role in Rab1 trafficking towards the Golgi complex. The exchange rate of Golgi resident proteins between peripheral Golgi stacks is too slow ($\sim 2 \text{ h}$ for equilibration)⁴¹ to account for the fast dynamics of Rab1 trafficking ($t_{1/2} = 70 \text{ s}$). Presumably, Rab1 trafficking under this condition can be attributed to GDI-mediated recycling, a non-vesicular transport pathway. Since the majority of expressed Rab1 molecules in cells are prenylated (**Fig. S16**), the prenylated Rab1 either binds to GDI/REP or associates with membranes. According to our previous measurements on Rab1 activity in the cytoplasm, there are approximately 83% active GTP-bound Rab1 and 17% inactive GDP-bound Rab1.⁴² It is conceivable that most active Rab1 molecules are associated with vesicles and most inactive Rab1 are bound to GDI. The delivery of Rab1 to the Golgi consists of vesicular transport and non-vesicular transport (GDI). Therefore, $k_{\text{on}} = 0.83 \cdot k_{\text{on}}(\text{ves}) + 0.17 \cdot k_{\text{on}}(\text{GDI})$. k_{on} and $k_{\text{on}}(\text{GDI})$ were obtained via the experiments shown above. We can derive the rate constant for the delivery of Rab1 to the Golgi via vesicular transport $k_{\text{on}}(\text{ves}) = 1.9 \times 10^{-2} \text{ s}^{-1}$.

The rate for Rab1 trafficking from the Golgi to the cytoplasm in nocodazole-treated cells ($k_{\text{off}} = (0.26 \pm 0.1) \times 10^{-2} \text{ s}^{-1}$) is identical to that in untreated cells (**Fig. 3 B, Fig. S15 E and F, Movie S9**). This indicates that Rab1 trafficking from the Golgi largely depends on GDI-mediated retrieval.

GDF plays an important role in Rab1 spatial cycling

PRA1 is the mammalian homologue of yeast Yip3, which was the first protein shown to exhibit GDF activity¹⁰. To assess the role of PRA1 in Rab1 trafficking, we performed FRAP and FLAP measurements in PRA1 knockdown cells (**Fig. S17**). Interestingly, the subcellular localization of Rab1 was maintained in PRA1 knockdown cells (**Fig. 4 E and H**). However, the rate of Rab1 delivery to the Golgi was significantly reduced ($k_{\text{on}}(\text{FRAP}) = (0.8 \pm 0.2) \times 10^{-2} \text{ s}^{-1}$ and $k_{\text{on}}(\text{FLAP}) = (0.7 \pm 0.1) \times 10^{-2} \text{ s}^{-1}$) (**Fig. 3 A, Fig. S18 A-D**). The kinetics of Rab1 trafficking from the Golgi did not significantly change in PRA1

knockdown cells with $k_{\text{off}} = (0.31 \pm 0.03) \times 10^{-2} \text{ s}^{-1}$ (**Fig. 3 B, Fig. S18 E and F**). These findings suggest that PRA1 plays an important role in Rab1 membrane delivery but not in retrieval of Rab1 from the membrane.

Mathematical simulations of Rab1 spatial cycling

To obtain a comprehensive picture of Rab1 spatial cycling, we set out to produce a mathematical model for the overall flux of Rab1 between the cytoplasm and the Golgi compartment. After fixing relevant model parameters by referencing them against experimental findings for the subcellular Rab1 distribution (**Fig. 4 H**) and relative amount of active Rab1 vs. inactive Rab1.⁴² we used this model to simulate experiments.

The most relevant model parameters and Rab1 trafficking processes are depicted schematically in **Fig. 5 A** (a complete list of parameters and simulation approaches can be found in the Supporting Information, Table S7). The model is based on a simplified cellular geometry with a circular Golgi ($r = 2.5 \mu\text{m}$) in a larger cytoplasmic compartment ($r = 10 \mu\text{m}$) (**Fig. 5 B**). Rab1 molecules either diffuse in the cytosol in complex with GDI, bind to vesicles in the cytoplasm, or are attached to the Golgi compartment. GDI-bound Rab1 is allowed to freely diffuse in the cytoplasm with a diffusion constant of $D = 10 \mu\text{m}^2/\text{s}$. Movement of membrane-bound Rab1 is assigned a diffusion coefficient of $d = 0.1D$ on the Golgi membrane⁴³. Nondirectional movement of Rab1-bound vesicles is described with $D_w = 0.1 \mu\text{m}^2/\text{s}$ ^{44, 45}. We assume that only active Rab1 is bound to vesicles that traffic along microtubules. The parameter for the overall speed ($J_a = 0.02 \mu\text{m}/\text{s}$) is lower than the upper limit set by previously reported values of instantaneous velocity of vesicular transport.^{46, 47}

To determine the parameters describing the Rab1 outflux from (a_1 and a_4) and the influx to the Golgi (b_1 and b_4), we utilized the k_{on} and k_{off} rates obtained from the FRAP and FLAP experiments (**Fig. 3 A and B**). While the observed rate constants do not directly correspond to the respective parameters, a_1 and b_1 are connected to GDI-mediated Rab1 trafficking rates, while a_4 and b_4 represent kinetic parameters that are linked to the rate constants obtained for Rab1 trafficking via vesicular transport (see the Discussion in the Supporting Information). Subsequently, the parameter values were adjusted to produce a reasonable fit with the observed characteristics of Rab1 activity. To this end, we referenced the obtained distribution of Rab1 between the Golgi and cytoplasm as well as the ratio of active and inactive Rab1 within these two regions under equilibrium against the experimentally obtained values. The relative concentration of Rab1 on the Golgi versus the cytoplasm from the simulation is approximately 7:1 (**Fig. 5 B**), which is analogous to our experimental finding of approximately 5:1 (**Fig. 3 C**). Furthermore, the simulation yielded the activity of Rab1 on the Golgi (41.77% GTP-bound, 58.13% GDP bound) and in the cytoplasm (81.13% GTP-bound, 18.87% GDP-bound) (**Fig. 5 C**, Supporting Information), which is keeping with experimental results obtained using

Rab1 COSGA sensor.⁴² The rates for Rab1 deactivation by GAP and activation by GEF were derived from available kinetic parameters.^{23, 48} For a detailed description, see the Supporting Information.

Next, we performed *in silico* perturbations of the Rab1 cycling system and examined its consequence on Rab1 spatial distribution. In the first numerical experiment, we switched off Rab1 deactivation by setting k_{13} and k_{23} to 0. As expected, Rab1 remained mostly active at the steady state and the spatial distribution of Rab1 on the Golgi versus the cytoplasm reduced from 7:1 to 5:1 (**Fig. S6**). This *in silico* result recapitulates the experimental result obtained for the constitutively active Rab1Q67L mutant. The experiments showed that the Golgi/cytoplasm distribution drops from 5.8:1 for Rab1 wild type to 2.6:1 for Rab1Q67L (**Fig. 4 F**). When the GEF function was abrogated in the simulation by setting k_{11} and k_{21} to 0, we observed an opposite effect with a significant increase of Rab1 enrichment at the Golgi (Golgi/cytoplasm distribution of 12:1, **Fig. S5**). In three further numerical experiments we switched off active vesicular transport, GDF function or GDI-mediated recycling by setting the parameter $J_a=0$, $b_1=0$ or $a_1=0$, respectively. The impairment of vesicular transport (**Fig. S7**) and GDF (**Fig. S8**) led to a decrease of the Golgi/cytoplasm distribution of Rab1 to 3.3:1 and 3:1, respectively. While the elimination of GDI function caused a dramatic increase in Rab1 enrichment at the Golgi with Golgi/cytoplasm = 17.8:1 (**Fig. S9**). This is in keeping with our experimental finding that GDI-mediated retrieval plays a dominant role in Rab1 outflux from the Golgi (**Fig. 3B**) (see more discussions in Supporting Information).

Discussion

In this study, we have shown that the subcellular localization of Rab1 is highly dynamic with a continuous flux between the cytoplasm and the Golgi compartment. In the cytoplasm, Rab1 either binds to vesicles that move towards the Golgi along microtubules or forms a complex with GDI that freely diffuses in the cytosol. On the Golgi, Rab1 associates with the membrane via its C-terminal prenyl anchor. Rab1 spatial cycling is controlled by vesicular transport and GDI-mediated recycling. The former requires active GTP-bound Rab, as GTP-Rab1 is involved in the recruitment of tethering factors such as p115 and GM130. These proteins tether ER-derived vesicles to the Golgi membrane, which are required for transport to the Golgi. GDI-mediated transport on the other hand requires inactive GDP-bound Rab, because the affinity of GDI towards GDP-bound Rab is more than three orders of magnitude higher than for the GTP-bound protein⁹. As a result, Rab activation stabilizes membrane attachment by impeding GDI-mediated recycling. These observations lead to the notion that the spatial cycles of Rab1 may be coordinated by the GTPase cycle.

We have demonstrated that the GTPase cycle is the driving force for the spatial cycles. The GTPase cycle is regulated by GEFs, GAPs, GDI and GDF. Perturbations of these regulators impede the kinetics of Rab1 spatial cycling and consequently its subcellular distribution. In our experiments, we used constitutively active Rab1Q67L that is deficient in GAP and GDI regulation. We observed a significant

decrease in k_{on} for influx and increase in k_{off} for outflux from the Golgi. Because Rab1 trafficking to the Golgi *via* vesicular transport ($k_{on}(ves) = 1.9 \times 10^{-2} s^{-1}$) is faster than that *via* GDI-mediated recycling ($k_{on}(GDI) = (0.9-1.2) \times 10^{-2} s^{-1}$), one would expect an increase in k_{on} for constitutively active Rab1Q67L that mainly undergoes vesicular transport, which accounts for the increase of observed k_{off} . The decrease in k_{on} for Rab1Q67L suggests that a functional GTPase cycle is also required for vesicular transport.⁴⁹ Rab1Q67L induces the formation of large vesicles in the cytoplasm (**Fig. 4B**). This observation suggests that constitutively active Rab1 aberrantly mediates tethering and fusion of vesicles, which may impede vesicular transport to the Golgi. It has been shown that Rab1Q67L led to decrease of COPII membrane association-dissociation kinetics, which may interfere with vesicular transport to the Golgi⁵⁰. The simulation under the GAP “off” conditions recapitulates the phenotype of Rab1Q67L, showing a reduction of Rab1 localization at the Golgi.

We have shown that GEF is required for Rab1 localization (**Fig. 4C, D, H**). This is in keeping with previous studies that Rab proteins are mistargeted when their cognate GEFs are mislocalized or depleted^{30, 51, 52} and that Rab5 permanently locked in a defined nucleotide (active or inactive) state loses its correct membrane targeting.⁵³ Activation by GEFs can only stabilize Rab attachment to a specific membrane compartment. However, it cannot accelerate the dissociation of the stable Rab-GDI complex.⁹ Because of the high affinity of GDI towards prenylated Rabs and the accordingly slow spontaneous dissociation, it has been proposed that an additional factor, GDF, is necessary to assist in releasing and targeting the GDI-bound Rab to a specific membrane.^{8, 9} The function of GDF (PRA1) in Rab1 spatial cycling has been for the first time elaborated in this study. Although knockdown of PRA1 does not lead to significant change in the overall Rab1 distribution in the cell (Fig. 4A, C, F), which is consistent with the previous observation on Ypt7 in Yip3 knockout cells,⁵¹ significant decreases on the Golgi localization of Rab1Q67L and k_{on} of Rab1 were observed in PRA1 knockdown cells (Fig. 3A, 4D and 4F). Since no significant change was observed in k_{off} of Rab1 when PRA1 was knocked down, PRA1 is required for delivery of Rab1 to the Golgi but not for the retrieval of Rab1 from the Golgi, in keeping with its proposed GDF function, i.e. facilitating the release of Rab from the Rab:GDI complex.^{10, 11}

We examined the function of GDI in the spatial cycles of Rab1. Nocodazole treatment inhibits vesicular transport and thus allows to specifically assess GDI-mediated recycling. The unchanged k_{off} in the presence of nocodazole suggests that the Rab1 outflux from the Golgi is mainly mediated by GDI. Additionally, our mathematical simulation revealed that disruption of GDI function leads to a substantial increase in Rab1 enrichment at the Golgi (17.8:1 in compare to 7:1). According to our previous measurements of Rab1 activity in the cytoplasm,⁴² Rab1 influx to the Golgi is largely mediated through vesicular transport by active Rab1 (83%). Therefore, the overall kinetics of Rab1 influx to the Golgi ($k_{on} = (1.6-1.9) \times 10^{-2} s^{-1}$) is much faster than the kinetics of GDI-mediated delivery ($k_{on}(GDI) = (0.9-1.2) \times 10^{-2} s^{-1}$), due to the relatively higher rate for the delivery of Rab1 to the Golgi via vesicular

transport when compared to that via GDI-mediated pathway. The relatively slower rates of GDI-mediated pathway (see also $k_{\text{off}}(\text{GDI}) = 0.26 \times 10^{-2} \text{ s}^{-1}$) suggest that there are slow rate-limiting steps involved in GDI-mediated recycling, probably arise from the dissociation of Rab1:GDI complex and the detachment of prenylated Rab1 from the membrane.⁹

Supporting Information

Mathematical simulation and Figures S10-S18 are shown in the Supporting Information.

Acknowledgement

We thank Sven Müller for technical support in microscopy. We thank Roger Goody for proof reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft, DFG (grant No.: SPP 1623 and SFB 642), Behrens Weise Stiftung, European Research Council, ERC (ChemBioAP) and Knut and Alice Wallenberg Foundation to Y.W.W.

Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure 1. Rab1 trafficking from cytoplasm to Golgi.

(A) Dynamics of EGFP-Rab1 by FRAP experiments. The bleached area is circled in red. After bleaching the fluorescence intensity increases at the region of interest (ROI, dashed red line) on the Golgi was followed by time-lapse imaging. Scale bar: 20 μm . **(B)** Dynamics of paGFP-Rab1 by FLAP experiments. The area selected for photoactivation is indicated by the dashed blue line. After photoactivation, the fluorescence intensity increase at the region of interest (ROI, dashed red line) in the Golgi region was followed by time-lapse imaging. The inset shows the Golgi marker, mKate2-Giantin. Scale bar: 10 μm . **(C)** Plot of individual ($n = 5$, gray lines) and average (black circles, mean $\pm$ s.d.) FRAP profiles observed from (A). k_{on} was obtained as the average of individual k_{obs} values. k_{obs} was determined by fitting to a monoexponential function. **(D)** Plot of individual (gray lines) and average (black circles, mean $\pm$ s.d.) FLAP profiles observed from (B). k_{on} was obtained as the average of individual k_{obs} values. k_{obs} was determined by fitting to a monoexponential function. ($n = 5$)

Figure 2. Rab1 trafficking from Golgi to cytoplasm.

(A) Dynamics of paGFP-Rab1 by FLAP experiments. The area selected for photoactivation is highlighted (dashed blue line). After photoactivation the fluorescence intensity decrease at the Golgi was followed by time-lapse imaging. The inset shows the Golgi marker, mKate2-Giantin. Scale bar: 10 μ m.

(B) Plot of individual ($n = 6$, gray lines) and average (black circles, mean $\pm$ s.d.) FLAP profiles from (A). k_{off} was obtained as the average of individual k_{obs} values. k_{obs} was determined by fitting to a monoexponential function.

Figure 3. Rate constants for Rab1 dynamics under different conditions.

(A) Summary of the apparent rate constants for cytoplasm-to-Golgi trafficking (k_{on}) for Rab1 wild type, Rab1Q67L, Rab1 wild type in cells treated with nocodazole and in PRA1 knockdown cells. **(B)** Summary of the apparent rate constants for Golgi-to-cytoplasm trafficking (k_{off}) for Rab1 wild type, Rab1Q67L, Rab1 wild type in cells treated with nocodazole and in PRA1 knockdown cells. **(C)** Plot of individual (n = 6, gray lines) and average (purple squares, mean $\pm$ s.d.) FLAP profiles for Rab1Q67L in comparison to average FLAP profile for Rab1 wild type (black circles, mean $\pm$ s.d.). k_{on} was obtained as the average of individual k_{obs} values. k_{obs} was determined by fitting to a monoexponential function. **(D)** Plot of individual (n = 5, gray lines) and average (purple triangles, mean $\pm$ s.d.) FLAP profiles for Rab1Q67L in comparison to average FLAP profile for Rab1 wild type (black circles, mean $\pm$ s.d.). k_{off} was obtained as the average of individual k_{obs} values. k_{obs} was determined by fitting to a monoexponential function.

Figure 4. Subcellular distribution of Rab1 proteins in PRA1, TRAPPC4 knockdown cells and after nocodazole treatment.

Fluorescence image of HeLa cells expressing (A) EGFP-Rab1 and (B) EGFP-Rab1Q67L constitutively active mutant in the presence of scrambled siRNA. In the EGFP-Rab1Q67L cells, enlarged vesicles in the cytoplasm can be observed. Boxed area is enlarged in the inset. (C) EGFP-Rab1 and (D) EGFP-Rab1Q67L expressed in TRAPPC4 knockdown cells. (E) EGFP-Rab1 and (F) EGFP-Rab1Q67L expressed in PRA1 knockdown cells. (G) Following nocodazole treatment EGFP-Rab1 localizes to small Golgi fragments that are scattered throughout the cell and colocalize with the Golgi marker. The Golgi marker Giantin was used in all experiments. (H) Ratio of the fluorescence of EGFP-Rab1 proteins at the Golgi to cytoplasm in (A), (B), (C), (D), (E) and (F). WT: Rab1 wild type, QL: Rab1Q67L. Mean $\pm$ s.d. (n = 15). (I) Pearson's colocalization coefficient analysis of Rab1b proteins with the Golgi marker Giantin in the absence and presence of nocodazole. Two-tailed t-test: ***: $p < 0.001$, n.s.: not significant. Scale bars: 10 μ m.

Figure 5. Mechanistic model for Rab1 spatial cycling and overall distribution of Rab1 and activated Rab as determined through numerical simulation.

(A) Schematic representation of relevant parameters in the Rab1 cycling model. **(B)** Nondimensional overall Rab1 distribution in the cytoplasm and in the Golgi compartment. **(C)** The ratio of active GTP-bound Rab1 to inactive GDP-bound Rab1 in the cytoplasm and in the Golgi compartment.