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Guanine Nucleotide Exchange Factors (GEFs) Have a Critical but Not Exclusive Role in Organelle Localization of Rab GTPases*[S]

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Background: Rab localization has been ascribed to guanine nucleotide exchange factor (GEF) localization.
Results: GEF deletions result in mislocalization of Rabs to other membranes, which can be bypassed by a Rab mutant.
Conclusion: GEFs are critical for Rab localization, but Rabs also have a GEF-independent ability to localize correctly.
Significance: Our data reveal that both GEFs and Rabs contribute to Rab localization in cells.

Membrane fusion at eukaryotic organelles is initiated by Rab GTPases and tethering factors. Rabs in their GDP-bound form are kept soluble in the cytoplasm by the GDP dissociation inhibitor (GDI) chaperone. Guanine nucleotide exchange factors (GEFs) are found at organelles and are critical for Rab function. Here, we surveyed the overall role of GEFs in Rab localization. We show that GEFs, but none of the proposed GDI displacement factors, are essential for the correct membrane localization of yeast Rabs. In the absence of the GEF, Rabs lost their primary localization to the target organelle. Several Rabs, such as vacuolar Ypt7, were found at the endoplasmic reticulum and thus were still membrane-bound. Surprisingly, a Ypt7 mutant that undergoes facilitated nucleotide exchange localized to vacuoles independently of its GEF Mon1-Ccz1 and rescued vacuole morphology. In contrast, wild-type Ypt7 required its GEF for localization and to counteract the extraction by GDI. Our data agree with the emerging model that GEFs are critical for Rab localization but raise the possibility that additional factors can contribute to this process.

Organelles of the endomembrane system of eukaryotic cells are tightly interconnected. Transport of proteins and lipids between different organelles depends on vesicles that are formed at a donor membrane, released into the cytoplasm, and finally consumed by fusion with an acceptor organelle. To be able to fuse with the correct acceptor membrane, vesicles need to carry the right fusion machinery. In principle, three distinct machineries cooperate during fusion. For the initial association of vesicles with the membrane, Rab GTPases localize to vesicles and/or target membranes and recruit tethering factors. At the endosome-vacuole interface, the Rab Ypt7 functions together with the HOPS (homotypic fusion and vacuole protein sorting) tethering complex to promote the initial docking of membranes. The subsequent fusion depends on membrane-embedded SNAREs that are present on both membranes. SNAREs fold in a zipper-like manner into tight four-helix complexes. This brings the two membranes into close contact and thus mediates bilayer mixing (1–3). The main regulation of membrane fusion appears to occur at the level of Rabs and tethering proteins. Rabs are switch-like proteins that depend on helper proteins for nucleotide exchange and GTP hydrolysis (4–7). Guanine nucleotide exchange factors (GEFs)[2] convert GDP-bound Rab into the active GTP-bound state. Only in this active conformation do Rabs bind to their effectors, such as tethering factors. Due to their slow GTP hydrolysis rate, specific GTPase-activating proteins (GAPs) are required for efficient and fast GTP hydrolysis. They complement the active site of the Rab and increase the intrinsic hydrolysis rate by several orders of magnitude. Moreover, reversible attachment of Rabs to membranes requires prenylation of their C-terminal cysteines, which depends on Rab geranylgeranyltransferase and Rab escort protein (REP; Mrs6 in yeast). In the cytosol, prenylated Rabs are bound to the chaperone GDP dissociation inhibitor (GDI; Gdi1 in yeast), which is similar in sequence and structure to REP (8–10). REP and GDI interact with the switch regions, C-terminal residues, and the prenyl anchor of the Rab (8, 11), and both show preference for the GDP-bound form (12, 13). Next, a GDI displacement factor (GDF) may be required to load a Rab onto membranes (14, 15), followed by activation through a GEF. Yip3/Pra1 has been identified as a Rab9-specific GDF (16, 17). Like Yip3, other Yip family members are integral membrane proteins and interact with multiple Rabs in a prenylation-dependent manner. Yip proteins are considered as good candidates to control specific membrane targeting of Rabs (18). It has been shown, however, that the GEF activity of the *Legionella* protein DrrA/SidM is both necessary and sufficient to displace GDI and activate the host Rab1 protein independently of an additional GDF, at least in this system (19–21).

Within the endomembrane system, different Rabs act in consecutive fusion reactions. For exocytosis, Ypt31/32 is present on secretory vesicles. Recent data indicate that both the mem-

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[2] The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; REP, Rab escort protein; GDI, GDP dissociation inhibitor; GDF, GDI displacement factor; ER, endoplasmic reticulum; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

brane lipid phosphoinositide 4-phosphate and GTP-bound Ypt31 are necessary to recruit the downstream GEF Sec2 (22, 23). Consequently, Sec2 will recruit and activate the exocytic Rab Sec4 onto secretory vesicles (23). A similar scenario is found along the endocytic pathway. It is thought that GTP-bound Rab5 on early endosomes recruits the Mon1-Ccz1 complex (24, 25), which subsequently activates Rab7 (26). These data suggest that an activated upstream Rab, such as Ypt31 or Rab5 in the given examples, is necessary to recruit the GEF of the downstream Rab (6).

Interestingly, if GEFs were targeted to mitochondria, Rabs localized to this organelle as well, suggesting that GEFs determine the localization of Rab GTPases to their target organelle (27, 28). This raises the question as to whether cells require additional factors for correct Rab targeting. Here, we address this question by following the fate of selected Rabs upon manipulation of their respective GEFs.

EXPERIMENTAL PROCEDURES

Yeast Strains and Molecular Biology—The strains and plasmids used are listed in supplemental Tables S1 and S2, respectively. The *yip1-1* and *sec2-41* temperature-sensitive mutants were kindly provided by Dieter Gallwitz (Max-Planck-Institute, Göttingen, Germany) and Mary Munson (University of Massachusetts Medical School, Worcester, MA), respectively. The *bet3-1* and *trs130*(Δ*33*) temperature-sensitive mutants were kindly provided by Nava Segev (University of Illinois, Chicago, IL). Deletions and tagging of genes were done using homologous recombination of PCR fragments (29, 30). Ypt7 was cloned into plasmid pRS411-TPIpr-mCherry-V5, which was generated from plasmid pRS414-TPIpr-mCherry-V5 (a gift from Fulvio Reggiori, University Medical Center Utrecht, Utrecht, The Netherlands). The pVT100-dsRed plasmid containing a marker for the mitochondrial matrix was also provided by Fulvio Reggiori. Coding sequences of Rab7 and Sec4 were provided by Francis Barr (Molecular and Clinical Cancer Medicine, University of Liverpool, Merseyside, United Kingdom) and inserted into the pRS414-PHO5pr-dsRed and pRS415-NOP1pr-yEGFP plasmids, respectively. Point mutations in *YPT7* and *VPS21* were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Microscopy—Yeast cells were grown to mid-log phase in yeast extract/peptone/dextrose, yeast extract/peptone/galactose, or synthetic dextrose complete medium lacking selected amino acids or nucleotides; collected by centrifugation; washed once with synthetic dextrose complete or synthetic galactose complete medium supplemented with all amino acids; and immediately analyzed by fluorescence microscopy. For FM4-64 staining of vacuoles, cells were incubated with 30 μM FM4-64 for 30 min, washed twice with yeast extract/peptone/dextrose medium, and incubated in the same medium without dye for 1 h. Images were acquired with a Leica DM5500 B microscope equipped with a SPOT Pursuit camera with an internal filter wheel (D460sp, BP460-515, and D580lp, Leica Microsystems GmbH), fluorescence filters (49002 ET-GFP (FITC/Cy2), excitation ET470/40×, emission ET525/50m; Wide Green, excitation D535/50×, emission E590lp; 49008 ET-mCherry/Texas Red, excitation ET560/40×, emission ET630/75m; Chroma Technology Corp.), and MetaMorph 7 software (Visitron Systems, Munich, Germany). Images were processed using NIH ImageJ 1.42 and AutoQuant X v1.3.3 (Media Cybernetics, Inc.).

Cell Lysis and Membrane Fractionation—Yeast cells grown in yeast extract/peptone/dextrose or yeast extract/peptone/galactose medium to $A_{600} \sim 1$ were collected by centrifugation and treated with 10 mM DTT, followed by incubation with lyticase for 20 min at 30 °C. Spheroplasts were then resuspended in lysis buffer (200 mM sorbitol, 50 mM KOAc, 20 mM Hepes-KOH (pH 6.8), 1× protease inhibitor mixture (0.1 μg/ml leupeptin, 1 mM *o*-phenanthroline, 0.5 μg/ml pepstatin A, and 0.1 mM Pefabloc), 1 mM PMSF, and 1 mM DTT) containing 2 μg/ml DEAE-dextran and incubated on ice for 5 min, followed by a 2-min incubation at 30 °C. Lysates were centrifuged at 300 × *g* for 3 min at 4 °C, and the supernatant was then centrifuged at 13,000 × *g* for 15 min to generate P13 (pellet) and S13 (supernatant) fractions. The S13 fraction was centrifuged at 100,000 × *g* for 30 min to obtain P100 (pellet) and S100 (supernatant) fractions. TCA-precipitated supernatants and pellets were resuspended in SDS sample buffer, and proteins were separated by SDS-PAGE, followed by Western blotting and detection using antibodies against Ypt7, Vac8, GFP, or Arc1.

RESULTS

Rab GTPases Are Mislocalized in the Absence of Their GEFs or upon GEF Inactivation—Relocalization experiments have shown that GEFs can determine the localization of Rab GTPases to their target membrane (27, 28). Using the same strategy, we observed that the Rab Ypt7 remains associated with the vacuole membranes when its GEF Mon1-Ccz1 is targeted to mitochondria via the C-terminal transmembrane domain of the mitochondrial Fis1 protein (Fig. 1*A*). This suggests that Ypt7 is activated either by the mitochondrial GEF or by a small pool of endosome-localized GEF, which was not sorted to the mitochondria. As this approach was thus unsuccessful in yeast, we decided to use an alternative assay and followed the fate of the Rab closely in GEF deletion strains. We have previously used subcellular fractionation to analyze the distribution of Ypt7 in cells lacking its GEF but did not observe any significant change compared with the wild type (Fig. 1*B*) (26). In contrast, when we examined GFP-tagged Ypt7 by fluorescence microscopy, we noticed that Ypt7 was not found exclusively at the vacuoles in *mon1*Δ and *ccz1*Δ cells and accumulated at different membrane structures. This distribution pattern was also distinct from the multilobed vacuoles observed upon loss of the HOPS subunit Vps39 (Fig. 1*B*). This confirms that Rab effectors, such as Vps39, are downstream of the GEF. Similar membrane redistribution was also observed for mCherry-tagged Ypt7 in GEF deletion strains (supplemental Fig. S1*A*). It is thus unlikely that a potential GFP-induced oligomerization of GFP-Ypt7 affects the subcellular distribution of Ypt7. Human Rab7, which requires the human Mon1-Ccz1 complex for localization,[3] behaved like Ypt7 in GEF deletion strains (supplemental Fig. S1*B*).

[3] M. Cabrera, M. Nordmann, A. Perz, D. Schmedt, A. Gerondopoulos, F. Barr, J. Piehler, S. Engelbrecht-Vandré, and C. Ungermann, manuscript in preparation.

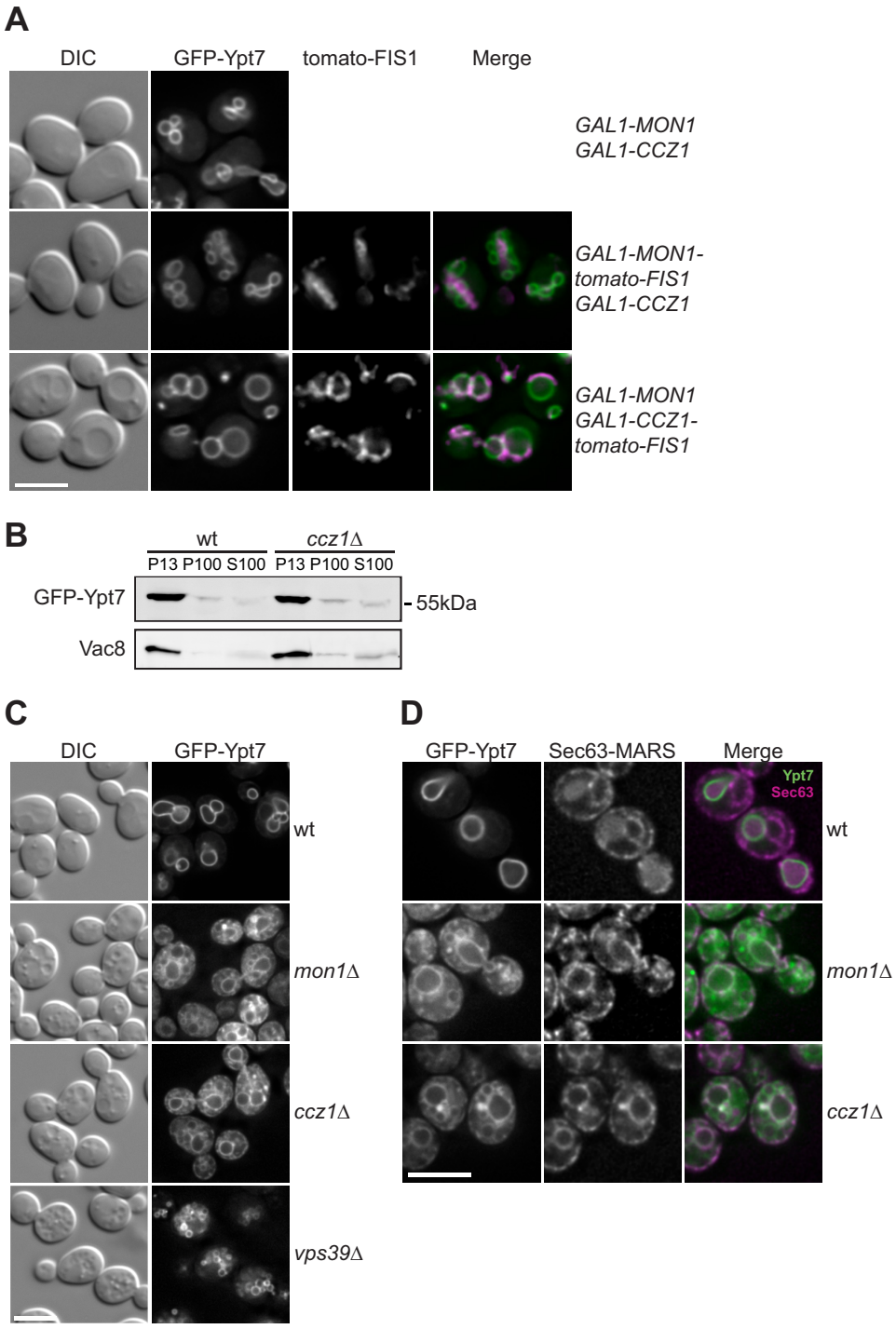

FIGURE 1. **Loss of the Mon1-Ccz1 complex alters Rab Ypt7 distribution.** *A*, localization of Ypt7 upon relocalization of the Mon1-Ccz1 complex to the mitochondria. *DIC*, differential interference contrast. *B*, subcellular fractionation of cells expressing GFP-tagged Ypt7 in wild-type (*wt*) and *ccz1*Δ cells. P13 is the pellet obtained after centrifugation at 13,000 × *g* for 15 min at 4 °C, and P100 and S100 are the pellet and supernatant obtained after centrifugation at 100,000 × *g* for 30 min at 4 °C, respectively. Protein distribution was analyzed by Western blotting and antibody decoration. Note that Vac8 was used as a vacuole marker. *C*, localization of GFP-tagged Ypt7 in the wild-type, *mon1*Δ, *ccz1*Δ, and *vps39*Δ strains. *D*, co-localization of Ypt7 and C-terminally mRFPmars-tagged Sec63 in the wild-type and GEF deletion strains. *Scale bars* = 5 μm.

To identify the membranes to which Ypt7 is targeted in the absence of the Mon1 and Ccz1 subunits, we followed several membrane markers and observed the best co-localization with the nuclear endoplasmic reticulum (ER) (Fig. 1*C* and supplemental Fig. S1*C*). We wondered whether this mislocalization is typical for Ypt7 or applies to other Rabs as well. For Ypt6, which operates between the endosome and Golgi, and the endosomal Rab Vps21, we observed similar redistribution upon GEF deletions. Ypt6 requires the two GEF subunits Ric1 and Rgp1 (31, 32), and loss of either subunit revealed partial mislocalization of Ypt6 to the ER, as observed for Ypt7 (Fig. 2*A*). For Vps21, it was shown recently that two GEFs, Vps9 and Muk1, act redundantly (33, 34). In agreement with this, only the dual deletion of both GEFs resulted in redistribution of Vps21 to the ER among

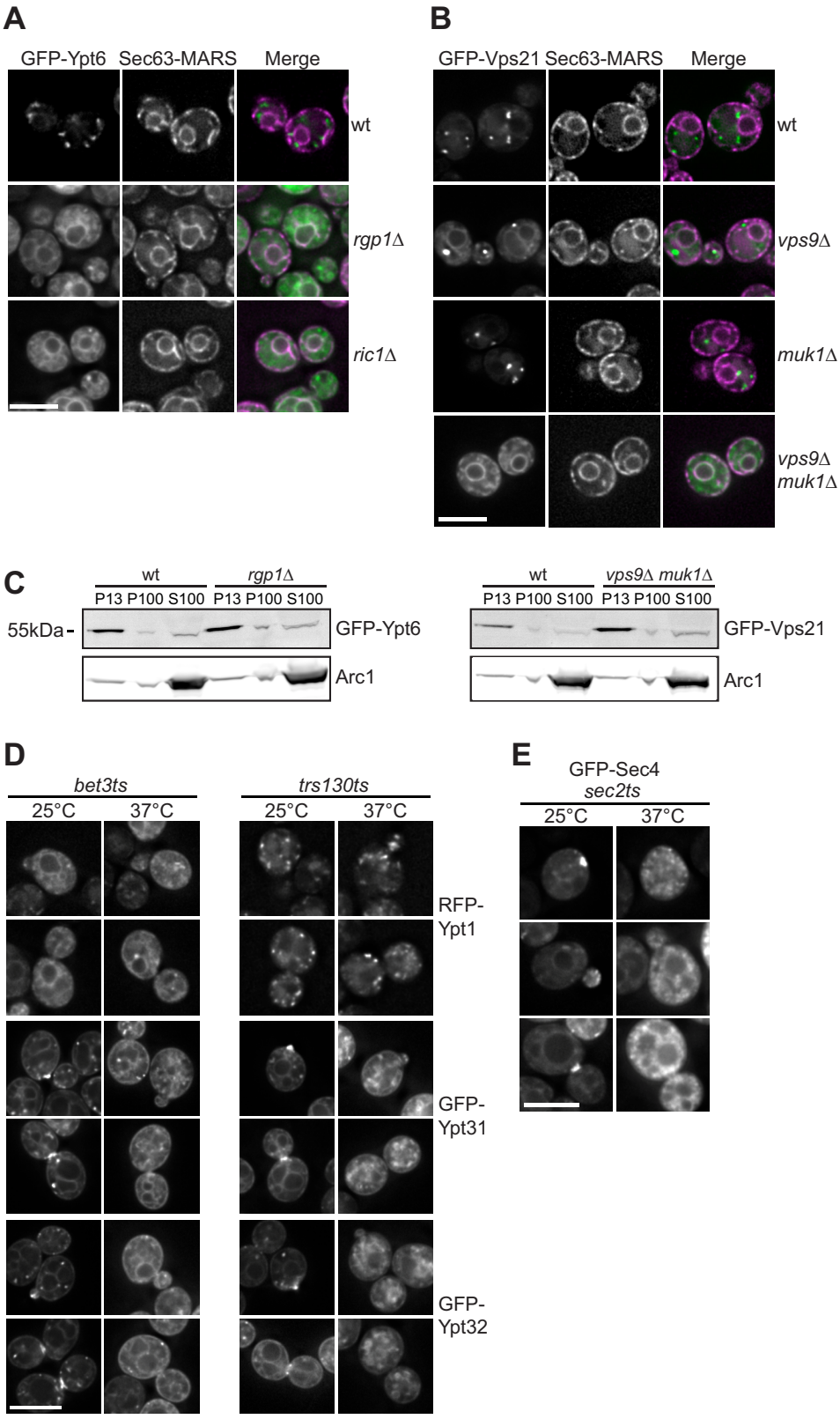

FIGURE 2. **GEF activity determines the correct localization of Rabs.** *A*, GFP-tagged Ypt6 distribution in the wild-type (*wt*) and GEF deletion strains. Sec63 was used as an ER marker. *B*, analysis of Vps21 co-localization with Sec63 in the wild-type and indicated deletion strains. *C*, GFP-tagged Ypt6 (*left panel*) and Vps21 (*right panel*) remained membrane-bound in the absence of their corresponding GEFs. Subcellular fractionation was performed as described for Fig. 1*B*. Arc1 was used as a cytosolic marker. *D*, subcellular targeting of Ypt1, Ypt31, and Ypt32 in the wild-type, *bet3*, and *trs130* temperature-sensitive (*ts*) mutants grown at 26 and 37 °C for 1.5 h. *E*, the localization of GFP-Sec4 was examined in the *sec2* temperature-sensitive mutant grown at 25 and 37 °C for 1 h, followed by fluorescence microscopy. *Scale bars* = 5 μm.

other membranes (Fig. 2*B*). As shown in Fig. 2*C*, both Rabs remained membrane-associated in the respective GEF deletion strains. We also tested the fate of exocytic Rabs, such as Ypt1, Ypt31 and Sec4, upon inactivation of their GEFs. Strikingly, the Golgi and polarized localization of Ypt31 and Ypt32 was reduced upon inactivation of the GEF complexes TRAPPI and TRAPPII by using the temperature-sensitive subunits *bet3* and *trs130*, respectively (Fig. 2*D*) (35). In contrast, Ypt1 Golgi distribution was affected only by inactivation of the TRAPPI complex (via the *bet3* temperature-sensitive mutant), even at permissive temperature (Fig. 2*D*) (35). For Sec4, a redistribution from a polarized localization to a more diffuse pattern was observed in the *sec2* temperature-sensitive mutant at different cell cycle stages (Fig. 2*E*), in agreement with previous findings (36). In summary, these data indicate that Rabs might remain membrane-bound even in the absence of their GEFs, but the targeting to the correct organelle requires the activity of the corresponding GEF.

Localization of Ypt7 to Vacuoles in the Absence of Its GEF—Elegant genetic studies revealed that deletion of Ccz1 can be bypassed by a point mutant in Ypt7 (37). Lysine 127 resides in the highly conserved GNKID (G4) motif, which recognizes the guanine base (Fig. 3*A*). The mutation K127E weakened the affinity for the nucleotide and may thus facilitate nucleotide exchange. We asked if this mutant could compensate for both GEF subunits or could rely on a partial GEF complex for function and thus followed the morphology of FM4-64-stained vacuoles as a readout (Fig. 3*B*). Expression of Ypt7 K127E, but not wild-type Ypt7, rescued the morphology of *mon1*Δ and *ccz1*Δ cells. Importantly, even the double mutant was rescued, indicating that the entire GEF complex is dispensable in this mutant. To follow the localization of Ypt7 K127E, we GFP-tagged the protein in wild-type, *mon1*Δ, *ccz1*Δ, and double deletion cells (Fig. 3*C*). Interestingly, Ypt7 was localized only to vacuoles and the cytosol. The additional fragmentation is likely due to the GFP tag on Ypt7, which partially impairs activity (38). In contrast, the GTP-locked Ypt7 mutant Q68L required a GEF for proper localization to the vacuoles (Fig. 3*D*). Our data indicate that the localization of Ypt7 to the vacuole membrane can be achieved also in the absence of its GEF, indicating that additional factors may support this process.

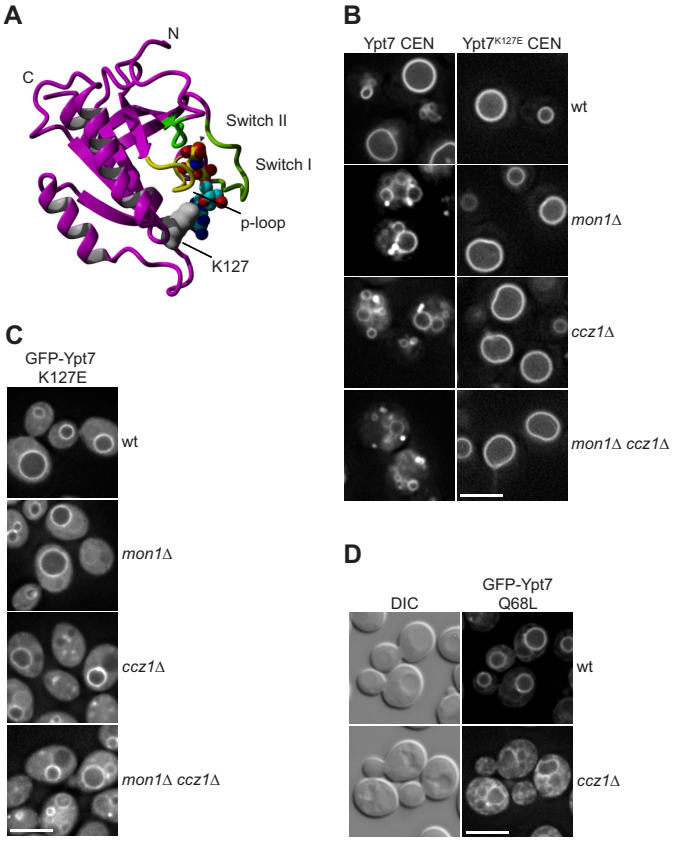

FIGURE 3. **Mutation K127E in Ypt7 bypasses the need for GEF activity.** *A*, representation of Ypt7-Gpp(NH)p (Protein Data Bank entry 1ky2; generated with YASARA), with the p-loop and switch regions indicated in *yellow* and *green*, respectively. The surface of Lys-127 is shown in gray. *B*, analysis of vacuole morphology via the lipophilic dye FM4-64 in the wild-type (*wt*) and GEF deletion strains expressing Ypt7 or the Ypt7 K127E mutant from a centromeric (*CEN*) plasmid. *C*, localization of the Ypt7 K127E mutant in wild-type cells and in cells lacking the Ypt7 GEF complex. *D*, microscopy of the GFP-tagged Ypt7 Q68L mutant in the wild-type and *ccz1*Δ strains. *Scale bars* = 5 μm. *DIC*, differential interference contrast.

Effect of Interactors and Modifiers on Rab Localization—We then asked if any of the previously identified Rab-interacting proteins might affect Ypt7 localization. The Yip family encodes Rab-interacting proteins, which localize to ER, Golgi, and endosomes and which might contribute to the specific distribution of Rabs. They have strong homology to mammalian Yip3/Pra1 (16) and have been postulated to act as GDFs at different organelles. Yip proteins might work in concert with other ER components, such as the reticulon Rtn1 and SNAREs (18, 39). Among the Yip family members, Yip1 is the only essential member due to its role in COPII vesicle formation (40). We thus analyzed the localization of Ypt7 to vacuoles, but we did not observe any change in the *yip1-1* mutant or in any other *yip* or *rtn1* deletion, including the quadruple mutant *yip2–5* (Fig. 4, *A* and *B*). We then asked if methylation of the C-terminal prenylation sequence might affect Rab localization. However, deletion of the prenylcysteine carboxyl methyltransferase Ste14 (41) did not interfere with localization of Ypt7, Vps21, and Ypt6 to their target organelles (Fig. 4*C*). Because all of these Rabs have a Cys-*X*-Cys tripeptide at their C termini, we wondered if replacement with a Cys-Cys sequence, which is not subject to methylation (42), would affect their distribution, but we did not observe any defect for Vps21 and Ypt7 (Fig. 4*D*). We thus consider it unlikely that Yip proteins or the prenylation motif plays a critical role in Rab localization.

The Mon1-Ccz1 GEF Drives Gdi1-bound Ypt7 to the Vacuole—Our data so far indicated that GEFs are critical for correct organelle localization of the tested Rabs but that additional factors might contribute to Rab localization, which become apparent only in Rab mutants. To monitor the cross-talk of Rabs with GEFs, GDI, and GAPs, we decided to focus on the membrane localization of Ypt7 *in vivo*. As mentioned above, Rabs cycle between the membrane-bound and active pool and the inactive GDP-bound fraction. Within the cytosol, newly synthesized Rabs are chaperoned by REP, which binds unprenylated and prenylated Rabs, and later on by Gdi1, which is the general Rab chaperone of the cytosol. We reasoned that overproduction of either of the two should increase the cytosolic pool of Rabs and thus followed Ypt7 as an example. Only overproduction of

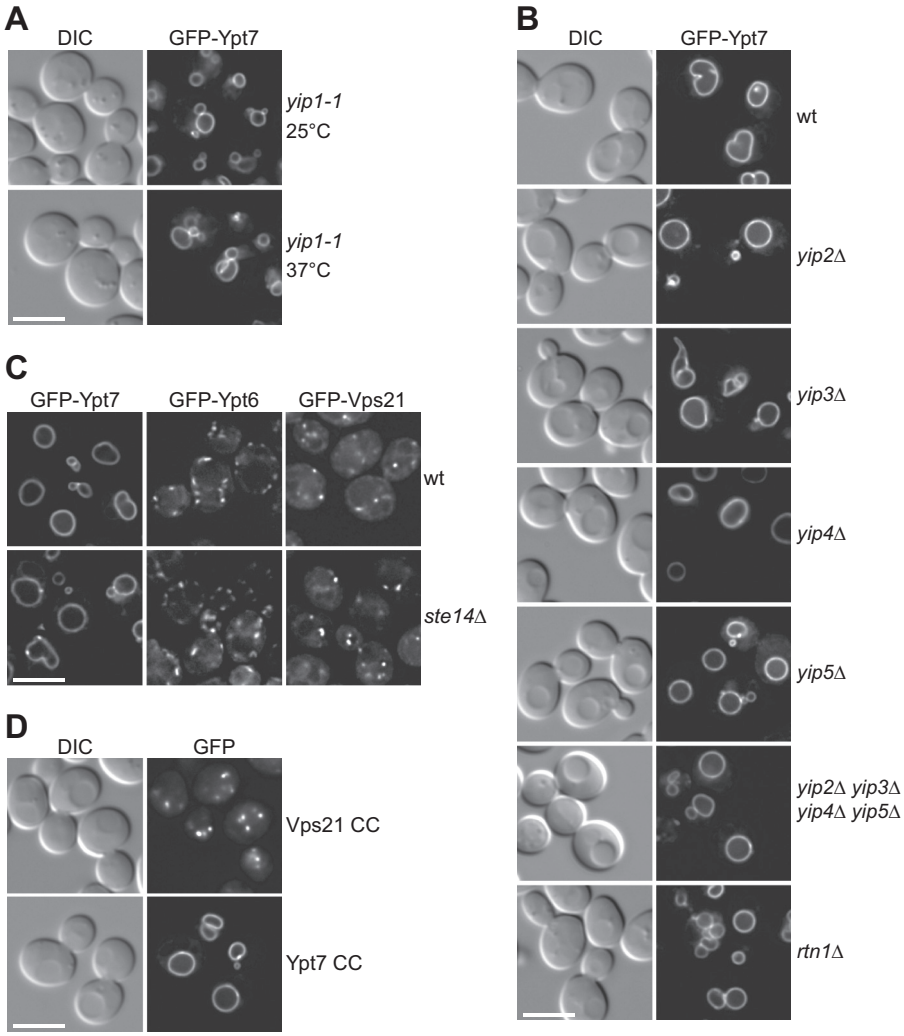

FIGURE 4. **Contribution of Yip proteins and methylation to Rab localization.** *A*, the *yip1-1* mutant carrying GFP-Ypt7 was grown at 25 and 37 °C for 1 h and analyzed by fluorescence microscopy. *B*, subcellular distribution of GFP-tagged Ypt7 in the indicated single and multiple deletion strains. *C*, microscopy of GFP-tagged Ypt7, Ypt6, and Vps21 was performed in the wild-type (*wt*) and *ste14*Δ strains. *D*, localization of Vps21 and Ypt7 mutants containing the C-terminal Cys-Cys (*CC*) prenylation motif. *Scale bars* = 5 μm. *DIC*, differential interference contrast.

Gdi1, but not that of the REP Mrs6, resulted in an increased pool of cytosolic Ypt7 (Fig. 5, *A–C*). Similar to the deletion of its GEF, Ypt7 was partially extracted from vacuole membranes and accumulated in the ER if either of its two known GAPs, Gyp7 or Msb3 (43–45), was overexpressed (Fig. 5*C*). This ER accumulation was not visible if both GAP and Gdi1 were co-overexpressed, suggesting that that ER pool is observed only if the binding capacity of Gdi1 is exceeded. In contrast, simultaneous overproduction of the GEF complex efficiently counteracted the excess of Gdi1 and deposited Ypt7 on the vacuole (Fig. 5*C*). This indicates that the GEF is the limiting factor needed to dissociate the Rab from Gdi1 and convert it to the membrane- and GTP-bound active form.

DISCUSSION

Our data reveal that GEFs are critical for the localization of Rab GTPases. In their absence or upon inactivation of the respective GEFs, Rabs lose their characteristic distribution pattern. For instance, Ypt7, Ypt6, and Vps21 prominently mislocalize to the ER. This indicates that Rabs might not become cytosolic if they cannot be localized to the right organelle. Our data indicate that Rabs have additional possibilities for targeting to the appropriate organelle membrane even in the absence of the GEF. For Ypt7, we have shown here that a mutation in the guanine base-binding motif not only rescues Ypt7 function in the absence of the entire GEF complex but also results in localization to vacuoles. Importantly, the wild-type protein requires the GEF for vacuole localization.

Recently, a few studies employed the mistargeting of GEFs to the mitochondrial outer membrane to demonstrate that GEFs are an important determinant for Rab localization (27, 28). These observations are in agreement with findings on the *Legionella* DrrA protein, which has strong GEF activity for Rab1. DrrA can displace Rab1 from the GDI-Rab1 complex due to its GEF activity and thus redirect ER membrane traffic during pathogen infection in cells (19–21). It is thus likely that GEFs have a critical role in GDI displacement and could thus drive Rab recruitment. However, one should keep in mind that the relocalization is likely also promoted by the large excess of the GEF on the mitochondrial surface, which could bypass any sup-

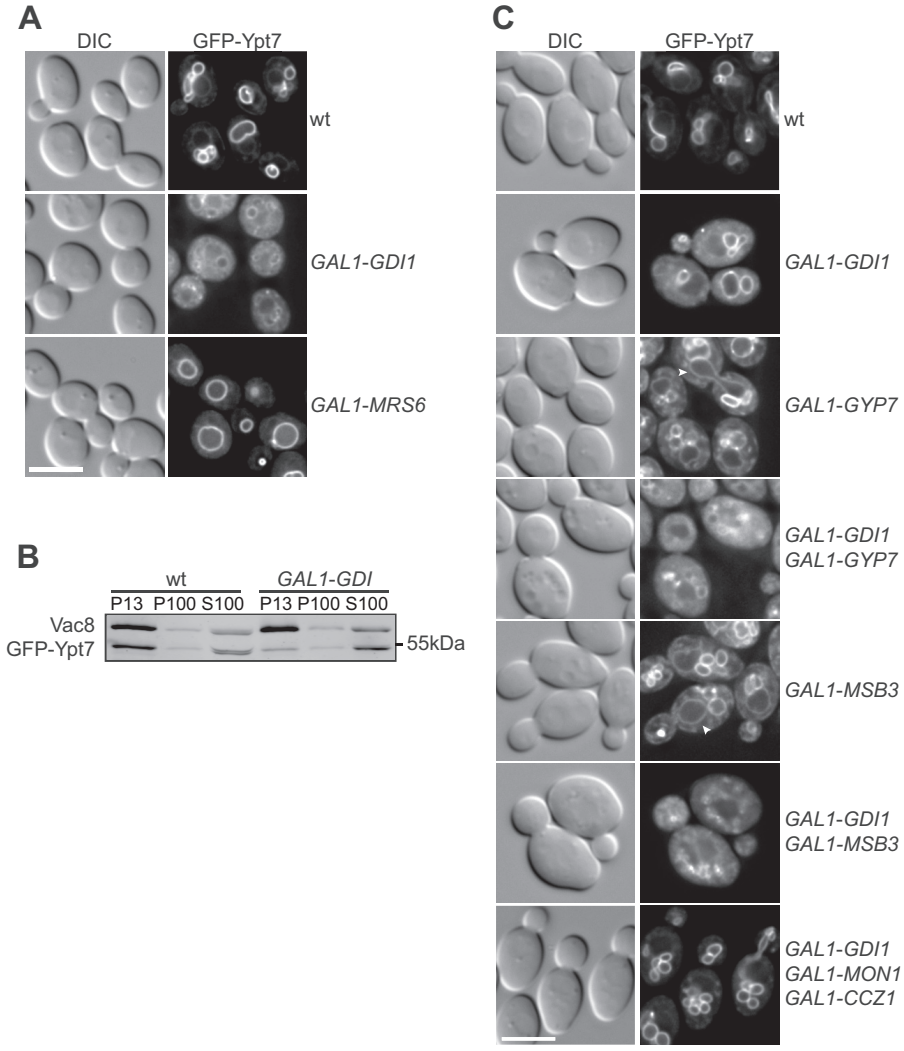

FIGURE 5. **Mon1-Ccz1 GEF activity for Ypt7 counteracts GDI.** *A*, membrane targeting of GFP-tagged Ypt7 was monitored in wild-type cells (*wt*) and in cells expressing Gdi1 or Mrs6 under the control of the *GAL1* promoter. *B*, subcellular fractionation of cells expressing GFP-Ypt7 and Gdi1 under the control of the *GAL1* promoter. The assay was performed as described for Fig. 1*B*. Vac8 was used as a vacuole marker. *C*, localization of GFP-Ypt7 in diploids cells overexpressing Gdi1 together with the Ypt7 GAPs (Gyp7 and Msb3) or Mon1 and Ccz1 was analyzed by fluorescence microscopy. ER labeling is indicated by *white arrowheads* in cells overexpressing the Ypt7 GAPs. *Scale bars* = 5 μm. *DIC*, differential interference contrast.

porting factor on the original membrane. In this context, it was surprising that correct targeting of Ypt7 was possible even if the entire GEF complex was lacking. This suggests that the GEF complex is not the sole determinant and that additional factors contribute to Rab targeting. As Ypt7 K127E is a fast cycling mutant, it is possible that the apparent targeting is partially driven by binding to effectors, such as the HOPS complex. Alternatively, additional cofactors may support the GEF also in wild-type cells. At this point, we can exclude that the proposed GDF proteins of the Yip family contribute to this targeting (Fig. 4), although it is possible that they act redundantly with other factors. At least the *yip2–5* quadruple mutant did not affect Ypt7 targeting. However, considering that wild-type Ypt7 relies strongly on the Mon1-Ccz1 complex for its targeting to vacuoles and that this complex can compensate for Gdi1 overexpression, we believe that the GEF is the most critical factor for organelle targeting of any Rab.

To test the mistargeting of Rabs to mitochondria *in vivo*, we also re-localized Mon1-Ccz1, but we did not observe any relocalization of the Rab. We speculate that the possible contacts between endosomes/vacuoles and mitochondria in yeast may allow Ypt7 targeting to vacuoles, and we cannot exclude that a minor portion of Mon1-Ccz1 escapes mitochondrial import and is still found on endosomes. It will therefore be important to dissect the precise GEF function of Mon1-Ccz1 in future assays to clarify if additional factors are important to support the GEF complex.

In light of our findings, how do we imagine the Rab cycle? Once a Rab is synthesized, it will be deposited by REP to the target organelle and then cycle in its GDP-bound form between the membrane and the cytosol (GDI-bound). In agreement with this, overexpression of the REP Mrs6 did not affect Ypt7 localization, whereas Gdi1 did (Fig. 5*A*). Only after the appropriate GEF is recruited to the organelle membrane, presumably via the preceding GTP-bound Rab, will the Rab be displaced from GDI and activated. This may be facilitated by additional factors, although we did not observe any effect upon deletion of the Rab-interacting Yip or Rtn proteins. They could alternatively

control Rab prenylation or GDI loading, which would explain their strong interaction with many different Rabs and GDI (16, 46–49). Several Rabs were found at the ER if they could not be delivered to the appropriate membrane. Interestingly, the ER also constitutes the target organelle for monoprenylated Rabs (47, 50) and chimeric Rab proteins (51, 52). We do not yet know if the ER localization is also important during the normal lifetime of Rabs, although it shows that a large fraction of Rabs do not remain cytoplasmic if the GEF is missing. It is possible that under conditions in which Gdi1 becomes limiting, during cell cycle transitions or stress, the ER serves as a default buffer to maintain the remaining Rab pool functional in the cell. According to our data, Rab localization to the right organelle can be explained by the interplay of GDI, GEF, and GAP. With excess GDI, most of the Rab likely cycles continuously between the cytosol and organelle membrane if the GEF is present. With excess GAP and normal levels of GEF and GDI, the large GDP-bound Rab pool is transferred by GDI to the ER. Only sufficient levels of the GEF can then shift the Rab quantitatively to the correct organelle.

On the membrane, the active Rab will recruit effector proteins and promote membrane fusion. For the endosome and vacuole membrane, Mon1-Ccz1 likely activates Ypt7 at the late endosome, which in turn interacts with vacuole-localized HOPS (53). The GAP would then be required to sharpen the activity of the Rab along one pathway, which could be further controlled by the active downstream Rab. How activation and turnover of Rabs are then controlled will require further studies.

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