Chapter 4

An ancient RAB5 governs the formation of additional vacuoles and cell shape in petunia petals*

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Abstract

Homologous ("canonical") RAB5 proteins regulate endosomal trafficking to lysosomes in animals and to the central vacuole in plants. Epidermal petal cells contain small vacuoles (vacuolinos) that serve as intermediate stations for proteins en route to the central vacuole. Here we show that transcription factors required for vacuolino formation in petunia induce expression of RAB5a. RAB5a defines a previously unrecognized clade of canonical RAB5s that is evolutionary and functionally distinct from ARA7-type RAB5s, which act in trafficking to the vacuole. Loss of RAB5a abolishes vacuolino formation, which cannot be rescued by the ARA7-homologs, and reduces cell height, while constitutive RAB5a (over)expression promotes homotypic vacuolino fusion, resulting in oversized vacuolinos and increased cell width. These findings provide a rare example of how gene duplication and neofunctionalization increased complexity of membrane trafficking during evolution and suggest a mechanism by which cells may form multiple vacuoles with distinct content and function.
Introduction

Membrane traffic is an essential process in all eukaryotes to deliver proteins and other compounds to distinct cellular compartments and involves the formation of membrane vesicles that bud of from donor membranes and their subsequent fusion with target compartments. SNARE proteins and RAB GTPases are major factors that determine the identity of membranes vesicles/endosomes and their fusion with membranes from specific target compartments. SNAREs, RABs and other trafficking regulators are encoded by gene families that existed already in early eukaryotes. Since the separation of the animals and plants these families have expanded by independent gene duplications in both lineages, which is thought to have been important for the increased tissue-specificity and complexity of trafficking systems during evolution\textsuperscript{1-4}.

The secretory pathway is a major trafficking pathway that delivers proteins to the plasma membrane, or, when the protein has a specific vacuolar sorting domain, to the vacuole or lysosome. Although many plant cells contain a single vacuole, several cell types possess multiple vacuoles with different content and, apparently, different functions\textsuperscript{5-9}. Cells in the epidermis of flower petals are dedicated to attract animal pollinators by visual (color) and/or chemical (scent) cues and by providing a landing site. Epidermal petal cells of petunia contain besides the large central vacuole (CV) filled with anthocyanin pigments numerous small vacuolar compartments, called vacuolinos, which lack anthocyanins\textsuperscript{10}.

In petunia flowers a complex of transcription factors (MBW), which consists of the MYB protein ANTHOCYANIN2, the bHLH protein AN1 and the WD40 protein AN11, drives the expression of genes involved in anthocyanin synthesis in late stages of bud development, just prior to opening of the flower\textsuperscript{11-14}. Slightly later, when the bud has reached its maximum size and the flower opens, a related MBWW complex, consisting of the MYB protein PH4, AN1, AN11, and the WRKY factor
PH3, activates a (partially) distinct set of target genes, which drive the hyperacidification of the CVs in colored cells of the petal epidermis to confer a red-violet flower color\textsuperscript{12,15,16} and the formation of vacuolinos\textsuperscript{10}. The major target genes involved in vacuolar acidification, \textit{PH1} and \textit{PH5}, encode two interacting P-ATPases that reside in the CV membrane (tonoplast) and act as proton pump\textsuperscript{17,18}. However, no MBWW-regulated target genes involved in formation of vacuolinos have been identified thus far.

Vacuolinos represent a novel intermediate station for membrane proteins, like \textit{PH1}, \textit{PH5}, KCO1, and vacuolar SNAREs, and soluble proteins, such as Aleu-GFP, on route to the CV. In transient expression assays these proteins accumulate first in vacuolinos about 24 hours after transformation, before they arrive at the CV, after approximately 48 hours\textsuperscript{10}. The formation of vacuolinos in petunia is governed by the MBWW complex \textit{PH4-AN1-AN11-PH3}. In epidermal petal cells of \textit{an1}, \textit{ph3} and \textit{ph4} mutants, which lack vacuolinos, proteins move “directly” (within 24 hours after transfection of gene constructs) to the CV, as they do in petal mesophyll and leaf cells\textsuperscript{10}. The MBWW target gene \textit{PH1} is essential for the trafficking from vacuolinos to the CV, as in \textit{ph1} mutant petals vacuolar proteins accumulate in (enlarged) vacuolinos but do not move on to the CV\textsuperscript{10}. This suggests that both the formation of vacuolinos and the transport from vacuolinos to the CV is operated by a largely unknown set of factors that are encoded by MBWW-regulated genes, possibly in combination with more ubiquitous factors that operate in vacuolar targeting pathway(s) that are expressed in other tissues.

GTPases of the RAB family are regulators of endomembrane trafficking in all eukaryotes\textsuperscript{19-21}. Arabidopsis possesses two “canonical” RAB5s, RHA1 and ARA7 (also called RABF2a and RABF2b), which are orthologous to metazoan RAB5s, and a more distant relative, ARA6 (also called RABF1), belonging to a plant-specific clade of RAB5s\textsuperscript{22, 23}. ARA7, RHA1 and ARA6 share the same activator, the
Guanine nucleotide Exchange Factor (GEF) VPS9a, but have different functions. ARA7 and RHA1 localize to nearly identical populations of prevacuolar compartments (PVCs)/multivesicular bodies (MVBs) and are involved in vacuolar trafficking in leaf and root cells, whereas ARA6 resides on a distinct population of endosomes and mainly functions in the traffic between PVCs/MVBs and plasma membrane.

In this study we found that petals from opening petunia flower express three canonical RAB5 genes, RAB5a, RAB5a1 and RAB5a2. RAB5a2 is orthologous to the well-studied RHA1 and ARA7 from Arabidopsis, while RAB5a and RAB5a1 represent evolutionary distinct clades that are missing in Arabidopsis and thus not previously recognized. Evolutionary divergence is mirrored by profound differences in their transcriptional regulation, intracellular localization and biological function. Genetic evidence shows that RAB5a expression is activated by AN1, PH3 and PH4 and is required for the formation of vacuolinos.

**Results**

**Identification of three canonical RAB5 homologs in petunia**

Examination of RNA-seq data indicated that petunia petals express three canonical RAB5 genes, RAB5a, RAB5a1 and RAB5a2 (Fig. 1a and Supplementary Fig. 1a). RAB5a is primarily expressed in petals and in the vasculature of leaves and stems, and is strongly reduced in an1, ph3 and ph4 mutant petals (Fig. 1a, b and Supplementary Fig. 1b), suggesting a role in the vacuolino pathway. RAB5a1 and RAB5a2, by contrast, are not controlled by an1, ph3 or ph4 (Fig.1a, c, d) and are expressed in a broad range of tissues (Fig. 1f, g).

To examine the relationship of these petunia proteins with the well-studied Arabidopsis proteins ARA7 and RHA1, which are canonical RAB5s, and AtARA6, which represents a distinct plant-specific clade of proteins related to RAB5 (refs, 22, 78).
we retrieved highly similar proteins from genome databases and performed phylogenetic analyses. This revealed the petunia protein PhARA6 is the apparent ortholog of ARA6, which in this and other analyses is more related to RAB22 than to RAB5s, whereas RAB5a, RAB5a1 and RAB5a2 are canonical RAB5s that are apparently orthologous of animal RAB5s (Fig. 1h). The canonical RAB5s from plants proved more diverse than hitherto thought. RAB5a2 is apparently orthologous to ARA7 and RHA1 from Arabidopsis, whereas RAB5a1 and RAB5a represent two distinct clades. This division in three clades existed already in early angiosperms, because distantly related Eudicot species belonging to Asterids (e.g. petunia, Solanum lycopersicum), Rosids (e.g. Vitis vinifera, Medicago truncatula) and Ranunculales (Aquilegia coelula), as well as monocot species (e.g. Oryza sativa, Ananas comosus) all possess homologs of RAB5a, RAB5a1 and ARA7/RHA1 (Fig. 1h).

In basal plants, such as the bryophytes Physcomitrella patens and Sphagnum fallax, the liverwort Marchantia polymorpha and the algae Klebsormidium flaccidum, a Charophyte, Chlamydomonas reinhardtii and Volvox carteri, two Chlorophytes, no such diversification of RAB5s is found: their RAB5s are more similar to the angiosperm RAB5a homologs than to RAB5a1 or ARA7/RHA1 homologs. This suggests that proteins from RAB5a serve an ancient function that is well conserved, whereas RAB5a1 and ARA7/RHA1 may have acquired of new function that allowed them to diverge faster, or that RAB5a1 and ARA7/RHA1 homologs were lost many times. Such gene losses may account for the lack of canonical RAB5s in the lycophyte Selaginella moellendorfii and absence of RAB5a and RAB5a1 orthologs in Arabidopsis and related Brassicaceae.
Figure 1. Identification and characterization of RAB5 genes in petunia. (a) Transcript reads for RAB5a, RAB5a1 and RAB5a2 per million of transcripts from RNA-seq analysis of petals from an1, ph3, ph4 and wild type (WT) plants. Values are presented as mean ± SD. n = 2 biological replicates for each plant. 

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**RAB5a**

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**Figure 1.** Identification and characterization of RAB5 genes in petunia. (a) Transcript reads for RAB5a, RAB5a1 and RAB5a2 per million of transcripts from RNA-seq analysis of petals from an1, ph3, ph4 and wild type (WT) plants. Values are presented as mean ± SD. n = 2 biological replicates for each plant.
of the mutants (an1, ph3 and ph4); and 4 biological replicates for wild type (WT). (b-d) RT-qPCR analysis of (b) RAB5a, (c) RAB5a1 and (d) RAB5a2 expression in petals of an1, ph4, ph3 and wild type flowers at different developmental stages. Values (mean ± SD) are based on 2 biological replicates (each with 2 technical replicates) and are standardized based on PhRAN. (e-g) RT-qPCR analysis of (e) RAB5a, (f) RAB5a1 and (g) RAB5a2 in petals (developmental stage1 to 7), leaves, stems and roots of the wild type petunia line M1xV30. Values are presented as mean ± SD. n = 3 biological replicates × 2 technical replicates in all cases. (h) Phylogenetic analysis of RAB5 proteins from different organisms. The tree is built by Maximum-likelihood and branch support is indicated as percentage of 300 bootstraps if ≥ 50%. Green, red and yellow arrows mark RAB5 homologs from Arabidopsis, Petunia and Citrus, respectively. Distinct RAB clades are given different colors as indicated on the right. Source data are provided as a Source Data file.

**Petunia RAB5 homologs interact with the GEF PhVPS9a**

To examine whether the three canonical RAB5 types can interact with, and potentially compete for, the same GEF activator(s), we analyzed their capacity to bind to the petunia homolog of VPS9a (PhVPS9a). In GAL4-based yeast two-hybrid assays we observed little or no interaction between PhVPS9a-GAL4BD and GAL4AD fusions of the three wild type RAB5s from petunia, AtARA6 from Arabidopsis and its petunia homolog PhARA6 (cf. Fig. 1), which was expected as in yeast RABs are thought to be mostly in their active GTP-bound form\(^{24}\). Therefore we examined interactions of the GDP-fixed mutants RAB5a\(^{T24N}\), RAB5a1\(^{S24N}\), RAB5a2 \(^{S24N}\) and PhARA6\(^{S46N}\) with PhVPS9a, and found positive interactions in all cases (Fig. 2a). The GTP-fixed mutant RAB5a\(^{Q69L}\), by contrast, could not bind PhVPS9a.

To examine whether these interactions occur also in petunia petal cells, we used split-YFP-based Bimolecular Fluorescence Complementation (BiFC) assays. In protoplasts originating from the petal epidermis or petal mesophyll, which can be distinguished by the presence of absence of anthocyanin pigments in their CVs respectively\(^{32}\), co-expression of nYFP-RAB5a\(^{T24N}\) and cYFP-RAB5a\(^{T24N}\) fusion did not result in noticeable YFP fluorescence (Fig. 2b), indicating that spontaneous reconstitution of YFP is negligible in these cells. Oligomerization of nYFP-PhVPS9a and nYFP-PhVPS9a was readily detectable (Fig. 2c), and consistent with previous findings on AtVSP9a (ref\(^{33}\)). Consistent with the yeast two hybrid results, we observed that cYFP-PhVPS9a interacted with GDP-fixed nYFP-RAB5a\(^{T24N}\) but
not or much less, with GTP-fixed nYFP-RAB5a\textsuperscript{Q69L} or wild type nYFP-RAB5a in cells from the petal epidermis and mesophyll (Fig. 2d). We obtained essentially similar results with native and GDP-fixed forms of RAB5a1, RAB5a2, PhARA6, and as a control AtARA7 (Fig. 2e). These results indicate that the three distinct canonical RABs, as well as PhARA6, can be activated by, and possibly compete for, the same GEF activator(s).

Figure 2. Interactions of different RAB5a paralogs with PhVPS9a, the petunia homologue of guanine nucleotide exchange factor (GEF) VPS9a. (a) Yeast two hybrid assay indicate the interaction between GAL4\textsubscript{AD} fusions of wild type and mutant versions of RAB5s and a GAL4\textsubscript{BD} fusion of PhVPS9a respectively. (b-e) Split YFP assays confirming the interaction of RAB5 proteins with PhVPS9a in cells from petunia petals epidermis and mesophyll. Transformed cells are marked by (co-)expression of the plasma membrane protein RFP-SYP122. (b) Negative control showing that reassociation of nYFP and cYFP moieties fused to non-interacting proteins (RAB5a\textsubscript{T24N}) is negligible.
(n = 29 cells). (c) Positive control showing that PhVPS9a oligomerizes, like AtVPS9a (n = 21 cells). (d) PhVPS9a interacts with RAB5aT24N (n = 61 cells), but not, or much less, with RAB5a (n = 23 cells) or RAB5aQ69L (n = 19 cells). (e) Interaction of Petunia RAB5 homologues and Arabidopsis RAB5 (AtARA7) with PhVPS9a (n = 13 to 33 cells). Bars = 10 µm. Source data are provided as a Source Data file.

**Intracellular localization of RAB5s**

To study the intracellular localization of the three petunia RAB5s we used fusions to fluorescent proteins (FPs). In petunia, and presumably other species, a considerable number of FP fusion proteins undergoes cleavage, because of which the separated moieties containing the FP and the tagged protein may have different fates and localize to different compartments or undergo differential turnover. In such cases the FP fluorescence does not reliably reflect the whereabouts of the tagged protein, even when expression of the FP fusion protein efficiently complements a mutant phenotype. Therefore, we analyzed GFP-RAB5a, GFP-RAB5a1 and GFP-RAB5a2 by immuno-blot analysis and found that these fusion proteins remain intact in petal cells (Supplementary Fig. 1c). Together with the finding that, like native RAB5a, GFP-RAB5a efficiently rescues the formation of vacuolinos in a rab5a mutants, and causes an enlargement of vacuolinos in protoplasts and transgenic plants (see below), this indicates that GFP-RAB5a, GFP-RAB5a1 and GFP-RAB5a2 reliably reflect the trafficking and function of the native (untagged) proteins.

To assess the localization of RAB5a in wild type (M1×V30) petal cells, we transiently co-expressed RFP-RAB5a with PH5-GFP (Fig. 3a) or Aleu-GFP (Supplementary Fig. 2b), which are markers of respectively the tonoplast and the lumen of the CV and vacuolinos. In protoplasts containing anthocyanins, which originate from the petal epidermis, part of RFP-RAB5a accumulated in the cytoplasm, the membrane of vacuolinos, and on smaller membrane compartments (puncta) that also contain PH5-GFP and Aleu-GFP and might represent PVCs (Fig. 3a, Supplementary Fig. 2b). Cytoplasmic RFP-RAB5 is likely to be in the (inactive) GDP-bound state, whereas membrane-bound RFP-RAB5 is in the (active) GTP-
bound state\textsuperscript{36}. Also in protoplasts from the petal mesophyll, which lack anthocyanins and vacuolinos, RFP-RAB5a localized in puncta, partially overlapping with the puncta marked by PH5-GFP or Aleu-GFP (Supplementary Fig. 2a, b).

To compare the localization of RAB5a, RAB5a1 and RAB5a2 proteins, we transiently co-expressed fusion proteins tagged with RFP or GFP from the constitutive 35S promoter in wild type (M1×V30) petal protoplasts (Fig. 3b-d and Supplementary Fig. 3a-b). In epidermal petal cells GFP-RAB5a1 localized in the cytoplasm and small punctate compartments that do not overlap with RFP-RAB5a positive puncta or vacuolinos (Fig. 3b, d). RFP-RAB5a2 localized to the cytoplasm and to punctate compartments that are distinct from puncta containing GFP-RAB5a, which localized is a similar way as RFP-RAB5a (Fig. 3c-d). Interestingly, GFP-RAB5a1 and RFP-RAB5a2 label in epidermal petal cells also distinct populations of puncta, with little or no overlap (Supplementary Fig. 3c). In petal mesophyll cells, which lack vacuolinos, the same RAB5a, RAB5a1 and RAB5a2 fusions also accumulated in the cytoplasm and distinct populations of puncta (Supplementary Fig. 3a-b).

To determine the identity of the GFP-RAB5a positive puncta, we co-localized GFP-RAB5a and markers for the endoplasmic reticulum (ER) (RFP-KDEL, ref\textsuperscript{36}), cis-Golgi (ERD2-RFP, ref\textsuperscript{37}), trans-Golgi network/early endosomes (TGN/EE) (RFP-LeRAB11, ref\textsuperscript{38}), and PVCs/MVBs (RFP-AtVSR2, ref\textsuperscript{39} and CHERRY-BP80, ref\textsuperscript{40}). In epidermal petal cells we observed no co-localization of GFP-RAB5a and RFP-KDEL, ERD2-RFP, or RFP-LeRAB11 (Supplementary Fig. 4). Although both BP80 and AtVSR2 are widely used as markers for PVCs/MVBs, they label distinct but overlapping populations of compartments in epidermal petal cells (Supplementary Fig. 5a), as do distinct AtVSR paralogs in tobacco cells\textsuperscript{41}. GFP-RAB5a positive puncta partially overlapped with CHERRY-BP80 and RFP-AtVSR2 positive compartments (Supplementary Fig. 5b, c), suggesting that they are a subpopulation
of PVC-like compartments. Furthermore, GFP-RAB5a localized together with CHERRY-BP80 and RFP-AtVSR2 in vacuolinos (Supplementary Fig. 5a, b). Interestingly, CHERRY-BP80 and RFP-AtVSR2, or at least their FP-tags, seem to become soluble in vacuolinos, as they accumulate in the vacuolino lumen rather than the membrane. In petal mesophyll cells, where RAB5a is normally not expressed, we observed a partial co-localization of GFP-RAB5a and RFP-KDEL (Supplementary Fig. 4a), and with the PVC markers CHERRY-BP80 and RFP-AtVSR2 (Supplementary Fig. 5a, b), indicating that in mesophyll cells part of GFP-RAB5a is localized to the ER, or in close proximity of the ER, and to PVCs. It is unlikely that the association of RAb5a with the ER is caused by the absence of vacuolinos, as we found no co-localization of GFP-RAB5 and RFP-KDEL in epidermal cells from ph3 and ph4 petals, which lack vacuolinos (Supplementary Fig. 6).

In Arabidopsis the RAB5a2-ortholog ARA7 resides in PVCs/MVBs and expression of the constitutively active GTP-bound mutant AtARA7Q69L causes the formation of enlarged spherical structures that originate from homotypic fusion of PVCs/MVBs. To examine whether vacuolinos originate from a similar class of PVCs/MVBs we co-expressed GFP-AtARA7Q69L and RFP-RAB5a in petunia petal protoplasts. In epidermal petal cells most of GFP-AtARA7Q69L and RFP-AtARA7Q69L remained cytosolic and only a small fraction accumulated in a few punctate compartments, which were distinct from RFP-RAB5a or RFP-RAB5a positive compartments (Supplementary Fig. 7). Expression of AtARA7Q69L did not have a noticeable effect on the formation and size of vacuolinos or trigger the formation of other spherical structures.

AtARA6 and AtARA7-positive PVCs/MVBs become in Arabidopsis cells labeled within 30 min after addition of the endocytic tracker FM4-64, indicating that they are intermediates in an endocytic pathway. In petunia protoplasts from both the
petal mesophyll and the epidermis FM4-64 is also rapidly endocytosed and labels after 10 minutes numerous endosomes that are distinct from the GFP-RAB5a positive punctate compartments and vacuolinos (Supplementary Fig. 8). After 1 hour the punctate FM4-64-positive and GFP-RAB5a-positive compartments remained separate populations, while co-localization of FM4-64 and GFP-RAB5a now became evident on vacuolinos, which in time continued to increase.

These findings suggest that (1) the RAB5a-positive puncta in epidermal petals cells are PVC-like compartments, that are distinct from the PVC/MVBs involved in endocytic pathway or the “direct” AtARA7 dependent pathway to the CV and (2) that vacuolinos may be the station where the endocytic pathway and vacuolino pathway merge.
Figure 3. RAB5a recruits on vacuolinos and localizes distinctly from RAB5a1 and RAB5a2. (a-c) Confocal micrographs of wild-type petal protoplasts co-expressing (a) RFP-RAB5a and PH5-GFP, (b)
GFP-RAB5a1 and RFP-RAB5a, and (e) GFP-RAB5a and mRFP-RAB5a2. Co-localization, or lack thereof, in the micrographs is quantified in the scatterplots on the right ($r_p =$ Pearson's $r$; $r_s =$ Spearman's $r$). (d) Pearson's $r$ and Spearman's $r$ correlation coefficients for co-localization of RFP-RAB5a and PH5-GFP (n=13 cells), GFP-RAB5a1 and RFP-RAB5a (n=14 cells), GFP-RAB5a and RFP-RAB5a2 (n=10 cells). Box plot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; diamonds, outliers. Bars = 10 µm. Source data are provided as a Source Data file.

**Effects of constitutive RAB5a expression**

We noticed that vacuolinos labeled by transiently expressed GFP-RAB5a were often much larger than those in cells expressing other GFP-tagged proteins. To further investigate this, we transiently co-expressed native RAB5a with PH5-GFP from the 35S promoter in wild type (M1×V30) petal protoplasts. When expressed alone, PH5-GFP localized 24 hours after transformation on vacuolinos with a normal size (1-10 µm) and reached the CV after 48 hours (Fig. 4a), as described previously. When co-expressed with 35S::RAB5a, PH5-GFP localized after 24 hours in vacuolinos, which were larger than normal (>15 µm), while subsequent sorting to the CV was strongly delayed (Fig. 4b). In the same cells the sorting of the plasma membrane marker RFP-SYP122, was not affected by 35S::RAB5a. 35S::RAB5a did not have a noticeable effect on the trafficking of PH5-GFP to the CV in petal mesophyll cells either. This indicates that constitutive RAB5a (over)expression from 35S only inhibits the pathway from vacuolinos to the CV.

Next, we generated transgenic wild type (M1×V30) plants constitutively (over)expressing 35S::RAB5a ($RAB5a^{OE}$). When $RAB5a^{OE}$ flowers opened, most of the petal tissue had a blue-violet color and acquired its normal red-violet color only at later stages (Fig. 4c and Supplementary Fig. 9a). The blue-violet flower color is typical of $ph$ mutants and is caused by the reduced acidity of the CV, where anthocyanin pigments reside, due to impaired expression of the proton pumping PH1-PH5 complex in the tonoplast. Due to the reduced acidity of the CV (and other PH1-PH5 containing compartments, like vacuolinos) the acidity of crude homogenates from $ph$ mutant petals is also reduced. Homogenate pH of blue
violet $RAB5a^{OE}$ petals, by contrast, was similar to that of wild type petals (Fig. 4c), suggesting that reduced acidity of the CV is compensated by increased acidity of other compartments, such as vacuolinos.

Epidermal cells of wild type petals contain numerous vacuolinos, which accumulate preferentially in the conical tip\textsuperscript{10}, whereas those from $RAB5a^{OE}$ petals contain a lower number of vacuolinos that are increased in size, which pushed the anthocyanin-containing CV down, to leave the conical cell tip essentially colorless (Fig. 4d and 5a). In addition, the tip of epidermal cells in $RAB5a^{OE}$ petals had a rounder shape, associated with increased cell width at 4 μm below the tip, as compared to the more pointed tips of wild type cells (Fig. 5). In protoplasts from the $RAB5a^{OE}$ petal epidermis transiently expressed PH5-GFP arrived after 24 hours in the (enlarged) vacuolinos, but little or no PH5-GFP arrived at the CV after 48 hours (Fig. 4e), similar to wild type cells transiently expressing $35S:RAB5a$. To confirm this result we generated stably transformed plants expressing $35S:GFP-RAB5a$ and found that vacuolinos in the petal epidermis of those plants were much larger than vacuolinos in control plants expressing $35S:PH5-GFP$ (Fig. 4f, g). The prolonged presence of PH5-GFP (Fig. 4e), and endogenous PH1 and PH5, on vacuolinos and the reduced trafficking to the CV will delay the acidification of the CV, explaining the blue-violet petal color when the flower opens, and increase acidification of vacuolinos, explaining why the overall acidity of $RAB5a^{OE}$ petals is not altered.
Figure 4. Ectopic expressed RAB5a induces the formation of enlarged vacuolinos. (a) Transient expression of PH5-GFP labels vacuolinos with normal size (24 hours) and reaches tonoplast (48 hours) in wild type (M1×V30) petal protoplasts (n\textsubscript{epidermis-24h} = 97, n\textsubscript{mesophyll-24h} = 26 n\textsubscript{epidermis-48h} = 33 and n\textsubscript{mesophyll-48h} = 15 cells). (b) Transient expression of RAB5a in wild type (M1×V30) petal protoplasts induces the formation of enlarged vacuolinos (n\textsubscript{epidermis-24h} = 19, n\textsubscript{mesophyll-24h} = 3, n\textsubscript{epidermis-48h} = 32 and n\textsubscript{mesophyll-48h} = 10 cells). (c) Flower phenotype and pH of the petal extracts (n = 6 flowers) of wild type and transgenic RAB5a\textsuperscript{OE} plant, bars = 1cm. (d) Light microscopy images of petal epidermal cells from wild type and RAB5a\textsuperscript{OE}, bars = 20µm. (e) Confocal micrograph of protoplasts driven from petals of a transgenic RAB5a\textsuperscript{OE} plant, transiently transformed with PH5-GFP and RFP-SYP122 (n\textsubscript{epidermis-24h} = 50,
Effects of loss of RAB5a function

To further assess the function of RAB5a, we generated transgenic M1×V30 plants in which RAB5a was down-regulated by RNAi (Supplementary Fig. 9c, d). In addition, we identified among progeny of line W138 (ref. 44) a mutant allele, rab5a\textsuperscript{x2043}, in which a dTPH1 transposon disrupts the coding sequence 252 bp downstream of the ATG (Fig. 6a and Supplementary Fig. 10a) and strongly reduces the amount of RAB5a transcripts, without affecting RAB5a1 and RAB5a2 mRNA levels (Fig. 6b). Petals from rab5a\textsuperscript{x2043} homozygotes had a similar color as RAB5\textsuperscript{+/+} siblings in the same background (W138), but the epidermal cells lacked vacuolinos and had a reduced cell width and height (Fig. 6c-f). Also down regulation of RAB5a by RNAi reduced the height of the cell and increased the apical width (at 4 \( \mu \text{m} \) below the tip), but not the width further down (Fig. 5a-d). This suggests that RAB5a inhibition reduces the height of the conical tip, because the width at 4 \( \mu \text{m} \) below the tip now measures the width of “main body” of the cell rather than the base of the tip.
Figure 5. Enlarged vacuolino induced by ectopic expression of RAB5a affects dimension of petal epidermal cells. (a) Light microscopy photographs of petal sections, transmission electron microscopy (TEM) images and environmental scanning electron microscopy pictures (ESEM) from flowers of wild type, RAB5aOE and RAB5aRNAi plants. Scale bars for light microscopy are 20 µm, for TEM and model 5 µm, and or ESEM 50 µm. V = vacuolinos, EV = enlarged vacuolinos, CV = central vacuole. Red asterisks mark the tip of the cells. (b) EM micrograph depicting how cell dimensions how height, basal width and apical width (width at 4 µm below the cell tip) of epidermal cells were measured. (c) Dimensions of epidermal cells from different lines (height and basal width n>100 cells; apical width, n>80 cells). Box plot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range; diamonds, outliers. Statistical significance of differences were assessed by Student’s t test/Mann-Whitney Rank Sum test; p-value < 0.001, three asterisks; NS, not significant. (d) Simplified model of the effect of altered RAB5a expression on dimensions and shape of petal epidermal cells of the same genotypes as above. Source data are provided as a Source Data file.
Figure 6. Impairment in RAB5a affects dimension of petal epidermal cells. (a) Schematic representation of the \textit{rab5a}^{2043} allele. Exons are indicated by black rectangles, introns by a thin line, and the inactivating 283 bp dTPH1 transposon with a red triangle. (b) RT-qPCR analysis of the expression of RAB5a, RAB5a1, and RAB5a2 in \textit{rab5a}^{2043} and isogenic wild type petals at flower stage 6. Values presented as mean ± SD (n = 3 biological replicates × 2 technical replicates) are normalized based on reference gene \textit{PhRAN}. Statistical significance of differences were assessed by Student's \textit{t} test/Mann-Whitney Rank Sum test; \( p \)-value < 0.01, two asterisks; NS, not significant. (c) Stage 7 flowers of \textit{RAB5a}^{+/+} and \textit{rab5a}^{2043} plants. Scale bars = 1cm. (d) Light microscopy photographs of petal sections, transmission electron microscopy (TEM) images and environmental scanning electron microscopy pictures (ESEM) from flowers of line R182 (\textit{RAB5a}^{+/+}) and R182 individuals homozygous for the \textit{rab5a}^{2043} mutation. (e) Dimensions (height, basal width (n>100) and apical width (width of 4 \( \mu \)m from the cell tip, n>80)) of epidermal cells from different lines. Box plot elements: center line,
median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range; diamonds, outliers. Statistical significance of differences were assessed by Student’s t test/Mann-Whitney Rank Sum test; p-value < 0.001, three asterisks. (f) Simplify model of the effect of vacuolinos on dimensions and shape of petal epidermal cells of the same genotypes as above. Source data are provided as a Source Data file.

To investigate how \textit{rab5a}^{x2043} affects the sorting of vacuolar proteins, we transiently expressed PH5-GFP or Aleu-GFP in petal protoplasts isolated from \textit{rab5a}^{x2043} homozygotes. In this genetic background (W138) PH5-GFP and Aleu-GFP reached vacuolinos within 24 hours after transformation and the CV after 48 hours in \textit{RAB5a}^{+/+} cells (Fig. 7a-b), as previously observed for wild type cells in the M1×V30 genetic background\textsuperscript{10}. However, in epidermal petals cells of \textit{rab5a}^{x2043} siblings, PH5-GFP and Aleu-GFP labeled 24 hours after transformation punctate structures instead of vacuolinos, and only a small fraction reached the CV 48 hours after transformation. In petal mesophyll cells from \textit{rab5a} mutant and \textit{RAB5a} siblings, PH5-GFP reached the CV directly within 24 hours after transformation, as in wild type (Fig. 7a-b). We obtained similar results in the \textit{RAB5a}^{RNAi} knock down line (Supplementary Fig. 10b) and in lines containing the \textit{rab5a}^{x2043} allele in a distinct genetic background accumulating malvidin anthocyanins (Supplementary Fig. 10c, d). Thus, \textit{rab5a}^{x2043} specifically affects the vacuolino pathway in epidermal petal cells and has no effect on the “direct” pathway to CV that is active in other cells.
Figure 7. Vacuolino formation and delivery of proteins to the central vacuole is blocked in rab5a<sup>x2043</sup> mutant petal epidermal cells. (a) Confocal micrographs of RAB5a<sup>+/+</sup> and rab5a<sup>x2043</sup> petal protoplasts transiently expressing PH5-GFP and RFP-SYP122 (RAB5a<sup>+/+</sup>: n<sub>epidermis-24 h</sub> = 69, n<sub>mesophyll-24 h</sub> = 29, n<sub>epidermis-48 h</sub> = 31 and n<sub>mesophyll-48 h</sub> = 24 cells. rab5a<sup>x2043</sup>: n<sub>epidermis-24 h</sub> = 52, n<sub>mesophyll-24 h</sub> = 21, n<sub>epidermis-48 h</sub> = 122 and n<sub>mesophyll-48 h</sub> = 35 cells). The dotted white dotted line indicate the slice chosen for the scans of the intensity of GFP (tonoplast) and RFP (plasma membrane) shown on the right. (b) Confocal micrographs of transiently expressed Aleu-GFP in RAB5a<sup>+/+</sup> and rab5a<sup>x2043</sup> petal protoplasts (RAB5a<sup>+/+</sup>: n<sub>epidermis-24 h</sub> = 35, n<sub>mesophyll-24 h</sub> = 16 cells, n<sub>epidermis-48 h</sub> = 28 and n<sub>mesophyll-48 h</sub> = 24 cells. rab5a<sup>x2043</sup>: n<sub>epidermis-24 h</sub> = 48, n<sub>mesophyll-24 h</sub> = 22, n<sub>epidermis-48 h</sub> = 53 and n<sub>mesophyll-48 h</sub> = 21 cells). Source data are provided as a Source Data file.

When we cotransformed rab5a<sup>x2043</sup> petal protoplasts with 35S:RAB5a along with 35S:PH5-GFP, we observed that transient RAB5a expression promptly restored the sorting of PH5-GFP to vacuolinos within 24 hours after transformation (Fig. 8a; Supplementary Fig. 10e). Transient expression of GFP-RAB5a rescued vacuolino formation with similar efficiency (Fig. 8b). Some of these rescued cells contained
enlarged vacuolinos and showed a delayed delivery of PH5-GFP to the CV, similar to wild type M1×V30 petal cells expressing 35S:RAB5a or 35S:GFP-RAB5a (Fig. 4b,e,g). Co-transformation of 35S:RAB5a1 or 35S:RAB5a2, by contrast, did not rescue PH5-GFP trafficking via vacuolinos (Fig. 8a), indicating that RAB5a1 and RAB5a2 are functionally distinct from RAB5a. Epidermal cells from ph3 and ph4 petals lack vacuolinos and transiently expressed proteins move directly (within 24 hours) to the CV. However, transient expression of RAB5a or GFP-RAB5a did not restore the formation of and/or the PH5-GFP trafficking via vacuolinos in ph3 and ph4 mutants (Supplementary Fig. 11). This indicates that the formation of vacuolinos requires, besides RAB5a, additional proteins encoded by other PH3/PH4 regulated genes.

We previously reported that mutations in PH1 block trafficking from vacuolinos to the CV and result in enlarged vacuolinos. To better position the function of RAB5a in the vacuolino pathway, we transiently expressed PH5-GFP in petal protoplasts from rab5a ph1 double mutants (Fig. 8c). In epidermal protoplast from rab5a ph1 petals PH5-GFP remains stuck on puncta, where it is still visible 48 hours after transformation (as in rab5a cells), instead of labeling vacuolinos and after 48 hours the CV (as in wild-type) or enlarged vacuolinos (as in ph1). Transient expression of RAB5a in rab5a ph1 petal epidermis protoplasts restored the presence of vacuolinos (rescue of the rab5a defect), but did not rescue the defect in trafficking to the CV caused by ph1. This indicates that RAB5a acts upstream of PH1 in the vacuolino pathway.
Figure 8. RAB5a cannot be replaced by RAB5a1 or RAB5a2 and functions upstream of PH1 in the vacuolino pathway. (a) Expression of 35S:RAB5a, 35S:RAB5a1 and 35S:RAB5a2 in protoplasts from petals of the rab5a<sup>x2043</sup> mutant. 35S:RAB5a, n<sub>epidermis-24h</sub> = 72 and n<sub>mesophyll-24h</sub> = 21 cells, n<sub>epidermis-48h</sub> = 51 and n<sub>mesophyll-48h</sub> = 16 cells. 35S:RAB5a1, n<sub>epidermis-24h</sub> = 48 and n<sub>mesophyll-24h</sub> = 21, n<sub>epidermis-48h</sub> = 33 and n<sub>mesophyll-48h</sub> = 17 cells. Images of 35S:RAB5a2, n<sub>epidermis-24h</sub> = 45 and n<sub>mesophyll-24h</sub> = 22 cells, n<sub>epidermis-48h</sub> = 68 and n<sub>mesophyll-48h</sub> = 25 cells. (b) Transient expression of GFP-RAB5a in rab5a<sup>x2043</sup> petal protoplasts (n<sub>epidermis-24h</sub> = 34 and n<sub>mesophyll-24h</sub> = 15 cells, n<sub>epidermis-48h</sub> = 27 and n<sub>mesophyll-48h</sub> = 8 cells. (c) Confocal images of rab5a<sup>x2043</sup> ph1<sup>+</sup> protoplast expressing 35S:RAB5a recruit PH5-GFP to enlarged vacuolinos (Images of +none, n<sub>epidermis-24h</sub> = 46, n<sub>mesophyll-24h</sub> = 9 cells, n<sub>epidermis-48h</sub> = 65 and n<sub>mesophyll-48h</sub> = 7 cells. Images of 35S:RAB5a, n<sub>epidermis-24h</sub> = 21 and n<sub>mesophyll-24h</sub> = 6 cells, n<sub>epidermis-48h</sub> = 37 and n<sub>mesophyll-48h</sub> = 4 cells of 48h). Scale bars = 10 µm in all panels.

To characterize the PH5-GFP-positive puncta in rab5a epidermal petal cells and gain insight in the origin of vacuolinos, we co-transformed rab5a petal protoplasts with constructs expressing PH5-GFP and RFP- or CHERRY-tagged markers for different cell compartments (Fig. 9). In these experiments PH5-GFP labeled in epidermal petals cells puncta, as in rab5a protoplasts expressing PH5-GFP alone,
indicating that none of the FP-tagged marker proteins altered PH5-GFP localization. We observed no co-localization of PH5-GFP with RFP-KDEL, ERD2-RFP, or RFP-LeRAB11 in either epidermal or mesophyll petal cells (Supplementary Fig. 12). However, we did observe (partial) co-localization of PH5-GFP with both CHERRY-BP80 and RFP-AtVSR2 (PVCs/MVBs marker) in a sub-population of puncta (Fig. 9). Together this indicates that PVCs/MVBs constitute at heterogeneous population of compartments, at least in the petal epidermis of petunia, and (ii) that the PH5-GFP positive puncta in epidermal cells of \textit{rab5a}^{x2043} petals represent a subset of PVCs/MVBs (Fig. 4d-e).
Figure 9. RAB5a promotes homotypic fusion of sub populations of PVC to form vacuolinos. Confocal pictures of protoplast derived from the RAB5a knock down mutant (RAB5aRNAi) transiently expressing PH5-GFP together with PVCs/MVBs markers (a) CHERRY-BP80 and (b) RFP-VSR2. Yellow arrowheads indicate co-localization, red and green arrowheads absence of co-localization. Scale bars = 10µm. (c) Quantification of Pearson's ($r_p$) and Spearman's ($r_s$) colocalization coefficient between PH5-GFP and different organelle markers ($n = 11$ to $15$ cells).
Discussion

Previous findings indicated that the MBWW transcription factor complex PH4-AN1-AN11-PH3, activates a novel tissue-specific trafficking pathway to the CV via vacuolinos\textsuperscript{10}. Here we identified the first MBWW target gene, \textit{RAB5a} that is involved in the formation of vacuolinos. RAB5a represents an ancient previously unrecognized clade of “canonical” RAB5s that diverged from the well-studied RHA1/ARA7 homologs by changes in its transcriptional regulation, intracellular localization, and biological function, providing a rare example in support of the hypothesis that the diversification and the increased complexity of membrane trafficking during evolution was facilitated by duplication and neo-functionalization of genes encoding key-factors like RAB GTPases, and SNAREs\textsuperscript{1-4}.

The finding that expression of RAB5a alone is necessary but not sufficient to send proteins to vacuolinos, or the specific PVC-like precursors thereof, indicates that other MBWW-regulated factors are involved. We hypothesize that some of these unknown factors act before RAB5a in the vacuolino pathway, at or close to the point where this pathway and the ubiquitous “direct” pathway to the CV diverge. Thus, in \textit{an1}, \textit{ph3} and \textit{ph4} mutants the vacuolino pathway is blocked at an early point from which proteins can be redirected into the direct pathway to the CV, whereas in \textit{rab5a} mutants proteins are upheld at a later “point of no return” (in small PVC-like compartments) from where they cannot be redirected anymore. Interestingly, RAB5a1 and RAB5a2 are also expressed in petals, both in wild type lines and in \textit{mbww} mutants. The observations that RAB5a1 and RAB5a2 localize to different compartments than RAB5a, and cannot rescue vacuolino formation in \textit{rab5a} mutants, or block vacuolino to CV transport, underlines that these RAB5s have different functions, but does not exclude that they function at some other point in the vacuolino pathway. Probably RAB5a2 is, like its orthologs in Arabidopsis (RHA1 and ARA7) and tobacco\textsuperscript{26, 28, 45}, involved in the direct pathway to the CV, which
operates in the petal mesophyll and the epidermis of an1, ph3 and ph4 petals. Given its distinct intracellular localization RAB5a1 may have a function different from RAB5a and RAB5a2 that remains to be determined.

Our findings suggest a model (Fig. 10) in which vacuolinos originate from the RAB5a-mediated fusion of a sub-population of PVCs to form larger structures. In epidermal protoplasts from wild type petals, we could indeed observe fusion events among PH5-GFP-labeled vacuolinos (data not shown). By extension, the formation of enlarged vacuolinos upon (over)expression of 35S:RAB5a may result from an abnormal number of fusion events among vacuolinos and/or their precursors. The latter indicates that a single cell may generate multiple vacuoles with distinct function, content and/or size solely by regulating the temporal expression of RAB5s without a need for specific sorting domains that send proteins to one vacuole or the other. Epidermal petal cells in young petunia flower buds (stage 1, 1-2 mm length) already contain a CV, which during further development to stage 5 (maximum size bud) accumulates a.o. anthocyanins. Expression of PH genes and their targets, such as RAB5a, sets in later when anthocyanin synthesis is ceasing, because of which the newly formed vacuolinos are largely devoid of anthocyanins and other compounds and proteins that are synthesized only in earlier stages. While in wild type petals vacuolinos remain much smaller than the CV, prolonged RAB5a expression results in larger vacuolinos, to become essentially an additional anthocyanin-less vacuole that co-exists with the older anthocyanin-containing CV.

While vacuolinos are an attractive model to study membrane trafficking by genetic approaches, their biological function remains unclear. Vacuolinos may affect optical features of the colored petal cells, which are important for the attraction of pollinators\textsuperscript{46-48}, by excluding the anthocyanin-containing CV from the conical tip and by altering cell dimensions. In Arabidopsis petals the orientation of cortical microtubules cells is important to give epidermal cells the correct domed shape\textsuperscript{49},

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and the formation of vacuolinos might add to shaping epidermal cells, possibly by providing mechanical force. However, vacuolinos might exist also in other cell types, suggesting additional functions, as RAB5a is also expressed in the vasculature of petunia leaves, and in Citrus fruits, where AN1 drives expression of PH1 and PH5, to hyperacidify the CV of juice cells\(^5\), as well as a RAB5 gene\(^5\) (Cs1g14330/orange1.1g029103m) that is homologous to petunia RAB5a (Fig. 1e).

Whether vacuolinos may serve as a sorting station, to prevent selected proteins from reaching the CV, is currently under investigation.

Our findings reported here and elsewhere\(^1\) suggest that further analysis of flower color mutants and additional AN1-PH3-PH4 regulated genes, is likely to uncover additional factors and mechanisms involved in membrane trafficking that could not be predicted by other methods.

**Figure 10.** Schematic representation of the RAB5amediate pathway for vacuolino formation. Blue arrows denote the “direct” canonical protein sorting pathway to CV observed in leaf cells, petal mesophyll cells, or petal epidermis cells from mutants with a defective MBWW complex. Orange arrows denote the vacuolino pathway in which RAB5a promotes fusions among PVC and pre-vacuolinos prior to the PH1-dependent delivery of proteins from vacuolinos to the CV. Overexpression
of RAB5a from the 35S promoter induces the formation of enlarged vacuolinos by promoting fusions among PVCs and vacuolinos and inhibiting further trafficking to the CV. The possible route of endocytosis for FM4-64 in petal epidermal cells generated from GFP-RAB5aOE plant is indicated by magenta arrows.

Methods

Plant material

Petunia line R182 (rab5a^x2043) is a transposon insertion mutant for RAB5a in the W138 line. The (transformable) F1 hybrid M1×V30 (wild type) was used to generate stable transformants. V23 (phl^v23) is a petunia line harboring a stable phl allele disrupted by a 7-bp transposon footprint in the coding sequence. The rab5a/phl double mutant was isolated by PCR screening of the F2 progeny from the cross R182 (rab5a^x2043)×V23 (phl^v23). A rab5a^x2043 mutant in a malvidin background was isolated from the F2 of the cross of R182 (rab5a^x2043) with the wild type line V30. Transgenic plants expressing 35S:RAB5a, 35S:GFP-RAB5a, 35S:PH5-GFP and plants expressing proRAB5a:GFP-GUS were obtained by Agrobacterium tumefaciens-mediated leaf disc transformation of the F1 hybrid M1×V30 (wild type). RAB5a^RNAi knock down line was generated by RNA interference with in F1 hybrid M1×V30 (wild type). All Petunia plants used in this work were grown in a greenhouse under normal conditions (19°C/30°C min/max, with cycles of 16/8 hours light/dark in summer, and with cycles of additional illumination of 15/9 hours light/dark in winter).

RNA extraction and RT-qPCR

Total RNA was isolated from petunia tissues using the TRizol reagent (Thermo Fisher Sceintific). Real-time RT-PCR was performed with an iTaq Universal Syber Green kit (Bio-Rad) using primers listed in Supplementary Table 1) and an Applied
Gene and constructs

RAB5a coding sequence (CDS) was amplified from M1×V30 (wild type) petal cDNAs with primer 7253 and 7254 containing attB1 and attB2 sites using Phusion® High-Fidelity DNA Polymerase; Purified RAB5a CDS was subsequently cloned into Gateway® pDONR™221 donor vector as an entry clone by Gateway® BP recombination reaction (Invitrogen™ Clonase™ Gateway™ BP Clonase II Enzyme Mix); Finally Gateway® LR recombination reaction (Invitrogen™ Clonase™ Gateway™ LR Clonase II Enzyme Mix) were performed with RAB5a CDS entry clone and multiple destination vectors (Supplementary Table 2) to make functional plasmids for several purpose. By using the same procedures, CDS of RAB5a1 (primer 7602 + 7603), RAB5a2 (primer 7607 + 7608), PhARA6 (primer 7577 + 7578) and PhVPS9a (primer 7508 + 7509) were amplified and subsequently subcloned into multiple destination vectors (Supplementary Table 2). Promoter sequence of RAB5a (primer 7513+7514) obtained from wild type petal gDNA with Phusion® High-Fidelity DNA Polymerase, cloned into Gateway® pDONR™221 donor vector, and recombined with destination vector pKGWFS7 to drive the expression of GFP and GUS. Constitutively active (GTP-bound) and constitutive negative (GDP-bound) mutants of RAB5s were generated by PCR based site-directed mutagenesis\textsuperscript{52}. The GDP-bound mutant of RAB5a termed RAB5a\textsuperscript{T24N} for example was generated as follows: Forward primer (7253), containing an attB1 site, and a reverse primer (7656) containing the desired mutation (mutate T (ACT) to N (AAT)) were used in a first round of PCR with Phusion® High-Fidelity DNA Polymerase. The resulting PCR product was used as forward primer together with reverse primer (7254), containing an attB2 site, in a second PCR reaction. Products of this second PCR reaction were cloned into the Gateway® pDONR™207 donor, sequenced and
subsequently transferred by Gateway recombination into multiple destination vectors for functional constructs (Supplementary Table 2). For RAB5a RNA interference (RNAi), a target sequence containing partial the last exon and 3’utr were amplified with primers (7854+7855), which were cloned into Gateway® pDONR™221 donor vector and pK7GW1WG2(I) binary silencing vector. 35S: PH5-GFP was identified as marker for vacuolinos membrane and tonoplast of CV; 35S: RFP-SYP122 has been described as plasma membrane (PM) marker; detailed description of how these plasmids were made published before. RFP-AtARA7Q69L, GFP-AtARA7Q69L and RFP-AtVSR2 are gifts from L.W. Jiang. RFP-KDEL, ERD2-RFP and RFP-LeRAB11(ref) were described in previous works. CITRINE-AtVSR2 is kindly provided by Dr. Nadine Paris. All the primers used in this study are reported in Supplementary Table 1. Vectors applied in this study are presented in Supplementary Table 2.

**Phylogenetic analysis and sequence alignment**

Amino acid sequences of RAB5 homologs from different plant species were selected from the Petunia Genome SGN dedicated database (http://petunia.sgn.cornell.edu/), Phytozome databases (https://phytozone.jgi.doe.gov/), PLAZA (https://bioinformatics.psb.ugent.be/plaza/), and Ensembl (http://www.ensembl.org/). Multiple sequences alignments were conducted by MUSCLE. The phylogenetic tree was produced by maximum-likelihood using the online tool PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/). G-blocks were applied to curate the alignment and 300 bootstrap replicates were used to assess the branch support. Amino acids sequence alignment was performed by ClustalX version 2.1, and further edited by program GeneDoc version 2.7.

**Protoplast transformation and confocal microscopy**
Protoplast isolation and transformation was done as described in previous work\textsuperscript{32}. Transfected cells were imaged with a Zeiss LSM510 confocal microscope using a 40x/1.2 water objective at 24 or 48 hours after transformation. For GFP and anthocyanins excitation we used a 488nm laser and detection filters BP505-550 and LP650 respectively. For RFP and CHERRY excitation was at 568nm and detection was achieved with a BP585-615 filter. Quantification of colocalization of FP tagged protein was performed with the PSC colocalisation plugin\textsuperscript{54} of the ImageJ. Protoplasts isolated from petals of a stable 35S:PH5-GFP transgenic line (PH5-GFP\textsuperscript{OE}) were loaded into WillCo-dish\textsuperscript{®} Glass Bottom Dishes (HBST-3522) to make movies of the fusion event between vacuolinos by Andor spinning disk confocal microscopy (Nikon TI microscope body equipped with Andor spinning disk and FRAPPA unit for confocal imaging).

**FM4-64 imaging**

Protoplasts prepared for staining with FM4-64 (SynaptoRed\textsuperscript{™} C2, Sigma-Aldrich\textsuperscript{®}) were isolated from petals of transgenic plants expressing 35S:GFP-RAB5a. After addition of FM4-64 to a final concentration of 50 µM FM4-64, protoplasts were kept in the dark and imaged after 10mins, 1 hour, 5 hour, and 24 hour with a Zeiss LSM510 confocal microscope using a 40x/1.2 water objective.

**Y2H and bimolecular fluorescence complementation (BiFC) assay**

Yeast two-hybrid assay was performed as reported before\textsuperscript{16}. In BiFC assay, plasmids encoding N-terminal fusion of protein of interest to nYFP and cYFP together with RFP-SYP122 were transformed into protoplast isolated from Petunia M1×V30 (wild type) petal. Transfected cells were imaged with a Zeiss LSM510 confocal microscope with a 40x/1.2 water objective at 24 hours after transformation. Over hundred transfected cells were observed for each independent experiment, and around 20 cells were imaged by confocal microscope.
Light-microscopy and transmission electron microscopy of petal sections

Semi-thin sections of petal tissue were prepared and observed as previously described\textsuperscript{10}. Transmission electron microscopy of petal cells was performed as previously reported\textsuperscript{18}. Petal epidermal cell dimension measurements were performed on the semi-thin sections of petal tissue. Height of cell was defined as the distance from the cell tip to the base of the cell. Basal width and apical width of cells were measured at the basal of the cell and at 4 µm down from the cell tip, respectively. All the measurements have been done with CellSens image analysis software.

Environmental scanning electron microscopy

Petal portions were fixed in 0.075M cacodylate buffer (pH 7.2) containing 3% (w/v) glutaraldehyde. Pre-fixed samples were then washed with 0.075M cacodylate buffer (pH 7.2) three times (7 mins each time), which were post-fixed in 0.075M cacodylate buffer (pH 7.2) containing 1% (w/v) OsO4 for 1 hour. Post-fixed samples were further washed three times (7 mins each time) in 0.075M cacodylate buffer (pH 7.2) and observed by ESEM (Environmental scanning electron microscopy) in wet mode\textsuperscript{55}.

GUS Staining

Flowers and leaves of petunia line transformed with proRAB5a:GFP-GUS and untransformed line(M1×V30) were collected and immediately dipped into cold 90% acetone for 20 mins. After removal of acetone by two washes with staining buffer (100mM sodium phosphate buffer (pH 7.2), 0.1% (w/v) Triton-X-100, 10mM EDTA) tissues were transferred to staining buffer containing 2mM X-Gluc (5-Bromo-4-Chloro-3-Indoyl-Beta-D-Glucuronide). To aid penetration of substrate into the tissue, sample were put under vacuum for three times 20 minutes and further incubated at 37 °C for several hours till the blue color developed and then transferred to 70% ethanol for long term storage and imaging.
**Western blotting**

GFP tagged proteins prepared for western blotting isolated from wild-type protoplasts transformed with plasmids for the expression of the different protein fusions. Protoplast isolation and transformation protocols\(^{32}\), as well as Western blotting\(^{18}\) procedures were previously reported.

**Statistical analysis**

Statistical analysis was performed using SigmaPlot 12.0. The normal distribution of values for all samples was confirmed by using Shapiro-Wilk normality test. Comparisons between two groups were performed using Student's t-test if variance was equal (Levene's mean test), or using Mann-Whitney Rank Sum test if variance was unequal.

**Data availability**

Sequence data generated in this study have been deposited in NCBI-Genbank and are accessible under accession numbers MH986793 (*RAB5a*), MH986794 (*RAB5a1*), MH986795 (*RAB5a2*), MK896357 (*PhARA6*), and MK896358 (*PhVPS9a*). A reporting summary for this article is available as a Supplementary Information file. The source data underlying Figures 1a-g, 2a, 3d, 4c, 4d, 5a, 5c, 6b-e, 7a, and 9c as well as Supplementary Figures 1b, 1c, 9d and 10a are provided as a Source Data file. Other data and biological materials are available from the authors on request.

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AtVSR2 constructs and to Dr Nadine Paris for providing CITRINE-VSR2. We are grateful to Pieter Hoogeveen and Bets Verbree for the care of the Amsterdam Petunia Collection.
Supplementary Figure 1. Petunia genome possesses 3 RAB5a paralogs. (a) Protein sequence alignment for RAB5a homologues from Petunia and Arabidopsis. Amino acids in the conserved motifs are highlighted in red. G, guanine-base binding motif; PM, phosphate/magnesium-binding motif. (b) Histological GUS activity staining in flowers at different development stages, leaves of an untransformed and a proPhRAB5a: GUS-GFP expressed line. Scale bars =1 cm. (c) Western blot of GFP fusion proteins in transgenic plants expressing genes of interests.
Supplementary Figure 2. Colocalization of RFP-RAB5a with PH5-GFP and Aleu-GFP. (a) Co-expression of RFP-RAB5a and PH5-GFP in mesophyll protoplast of wild type petunia (M1×V30) petal, 48 hours after transformation. (b) Petal protoplasts of a wild type co-transformed with RFP-RAB5a and Aleu-GFP. 48 hours after transformation. Scale bars = 10 µm.
Supplementary Figure 3. Colocalization of GFP-RAB5a1 with RFP-RAB5a and RFP-RAB5a2. (a) Co-expression of GFP-RAB5a1 and RFP-RAB5a in petal mesophyll protoplast of wild type petunia petal. (b) Co-expression of GFP-RAB5a and RFP-RAB5a2 in mesophyll protoplast of wild type petunia petal. (c) Confocal images of GFP-RAB5a1 co-expressed in wild type petal protoplasts with RFP-RAB5a2. Scale bars = 10 µm.
Supplementary Figure 4.
GFP-RAB5a does not co-localize with ER, cis-Golgi, and TGN/EE markers in protoplasts from Petunia petals. (a-b) Confocal micrographs co-expressing GFP-RAB5a with (a) RFP-KDEL, (b) ERD2-RFP and (c) RFP-LeRAB11. The scatterplots on the right show correlation of localization patterns, and are expressed as Pearson’s ($r_p$) and Spearman’s ($r_s$) correlation coefficients. Scale bars=10 µm.
supplementary Figure 5. Colocalization of GFP-RAB5a and the PVC/MVB markers BP80 and AtVSR2. (a) Co-expression of CHERRY-BP80 with CITRINE-AtVSR2 in wild type petal protoplasts. (b-c) Confocal micrographs co-expressing GFP-RAB5a (b) CHERRY-BP80, and (c) RFP-AtVSR2. The scatterplots on the right show correlation of localization patterns, and are expressed as Pearson’s ($r_p$) and Spearman’s ($r_s$) correlation coefficients. Yellow arrowheads indicate co-localization, red and green arrowheads absence of co-localization. Scale bars=10 µm.
Supplementary Figure 6. Colocalization of GFP-RAB5a and the ER marker RFP-KDEL in ph3 and ph4 mutant petal cells. Confocal micrographs of protoplast from (a) ph3 and (b) ph4 mutant petals transiently expressing GFP-RAB5a and RFP-KDEL 24 hours after transformation. The scatterplots on the right show correlation of localization patterns, and are expressed as Pearson's ($r_p$) and Spearman's ($r_s$) correlation coefficients Bars =10 μm.
Supplementary Figure 7. RAB5a does not co-localize with AtARA7Q69L in protoplasts from Petunia petals. (a) Confocal images of transiently expressed GFP-ARA7Q69L with RFP-RAB5a in wild type petal protoplasts. (b) Confocal images of transiently expressed RFP-ARA7Q69L with GFP-RAB5a in wild type petal protoplasts. The scatterplots on the right show correlation of localization patterns, and are expressed as Pearson’s ($r_p$) and Spearman’s ($r_s$) correlation coefficients. Scale bars=10 µm.
Supplementary Figure 8. FM4-64 positive puncta are different from GFP-RAB5a labeled compartment. FM4-64 staining of protoplasts isolated from *GFP-RAB5αOE* petals. Confocal images were taken at different time points after FM4-64 addition. Bars = 10 µm
Supplementary Figure 9. Phenotypes of transgenic loss and gain of function mutants for RAB5a.

(a) Color change during flower development in petals from stable transformants expressing 35S:RAB5a (RAB5aOE). (b) Confocal micrographs of leaf protoplast isolated from untransformed control (Wild Type, M1×V30) and RAB5aOE co-transfected with PH5-GFP and RFP-SYP122. (c) Flowers of Wild Type and RAB5aRNAi plants. (d) Expression data of RAB5a, RAB5a1 and RAB5a2 in stage 6 flower of RAB5aRNAi plants compared to Wild Type plants. Values are based on three biologicals replicates (for each biological replicate two technical replicates) and are standardized based on PhRAN, error bars represent standard deviation. Statistical significance of differences were assessed by Student’s t test/Mann-Whitney Rank Sum test; p-value < 0.01, two asterisks; NS, not significant. Scale bars= 1 cm in (a) and (c). Scale bars = 10 μm in (b).
Supplementary Figure 10. Effects of loss of RAB5a function in flowers with malvidin type anthocyanins. (a) Genomic PCR amplification of flanking sequences of the dTph1 transposon copy inserted in the RAB5a gene in a segregating population of R182 plants. (b) Confocal micrographs of protoplast from petals of RAB5a<sup>RNAi</sup> plants expressing PH5-GFP and RFP-SYP122. (c-e) Confocal micrographs of protoplast from petals of RAB5a<sup>−/−</sup> (c) and rab5a<sup>x2043</sup> (d), both progeny of the cross [rab5a<sup>x2043</sup> × V30] × S]. (e) Transient overexpression of 35S:RAB5a in protoplasts of rab5a<sup>x2043</sup> mutant, progeny of [rab5a<sup>x2043</sup> × V30] × S]. Scale bars of all confocal images, 10 µm.
Supplementary Figure 11. Forced expression of RAB5a does not rescue vacuolino formation in ph3 and ph4 mutant petals. Confocal micrographs of protoplast from ph3 and ph4 mutant petals (a) and (c), and the same cells transformed with 35S:RAB5a (b) and (d). Confocal images transiently expressed with GFP-RAB5a in petal protoplast of ph3 (e) and ph4 (f) mutant petal respectively. Bars =10 µm.
Supplementary Figure 12. No colocalization of PH5-GFP positive puncta and markers for ER, cis-Golgi and TGN/EE in RAB5a knock down mutant. Confocal pictures of protoplast derived from the RAB5a knock down mutant (RAB5a<sup>RAB5a<sub>KO</sub></sup>) transiently expressing PH5-GFP together with (a) RFP-KDEL, a marker for the ER, (b) ERD2-RFP, a marker for cis-Golgi membranes and (c) RFP-LeRAB11, a marker for the TGN/EE membranes. The scatterplots on the right show correlation of localization patterns, and are expressed as Pearson's (r<sub>p</sub>) and Spearman's (r<sub>s</sub>) correlation coefficients. Bars =10 µm
## Supplementary Table 1. Primers used in this study

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1 F indicates a forward orientation of the primer, relative to the orientation of the gene, and R reverse orientation

Sequence marked with green and red represent attB1 and attB2 respectively
### Supplementary Table 2. Recombinant vector and plasmids used in this study

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<td>Invitrogen™</td>
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Reference


