Abstract

The endolysosomal system fulfils a myriad of cellular functions predicated on regulated membrane identity progressions, collectively termed maturation. Mature or “late” endosomes are designated by small membrane-bound GTPases Rab7 and Arl8b, which can either operate independently or collaborate to form a joint compartment. Whether, and how, Rab7 and Arl8b resolve this hybrid identity compartment to regain functional autonomy is unknown. Here, we report that Arl8b employs its effector SKIP to instigate inactivation and removal of Rab7 from select membranes. We find that SKIP interacts with Rab7 and functions as its negative effector, delivering the cognate GAP, TBC1D15. Recruitment of TBC1D15 to SKIP occurs via the HOPS complex, whose assembly is facilitated by contacts between Rab7 and the KMI motif of SKIP. Consequently, SKIP mediates reinstatement of single identity Arl8b sub-compartment through an ordered Rab7-to-Arl8b handover, and, together with Rab7’s positive effector RILP, enforces spatial, temporal and morphological compartmentalization of endolysosomal organelles.

Keywords Arl8b; HOPS; Rab7; SKIP; TBC1D15

Introduction

The endolysosomal system consists of a dynamic network of vesicular structures working together to achieve controlled uptake and proteolysis of materials derived from the extracellular space. Regulated transport and sorting of cargoes along the endocytic route enable cells to effectively interpret and mitigate extracellular cues (Sigismund et al., 2012; Bakker et al., 2017), fight off pathogens (Gruenberg & van der Goot, 2006; Roche & Furuta, 2015) and sustain homeostasis (Lim & Zoncu, 2016). To ensure order within this vesicular network and regulate access to the proteolytic compartment, the endolysosomal system is compartmentalized in cellular space (Neefjes et al., 2017). Once nascent endosomes are acquired in the cell periphery, they undergo progressive maturation through controlled interactions with late compartments (Huotari & Helenius, 2011). This process of maturation is accompanied by ordered transitions in membrane identity (Cullen & Carlton, 2012), coupled to sequestration of cargoes marked for destruction onto intraluminal vesicles (ILVs; Christ et al., 2017). The resulting multivesicular bodies (MVBs; Woodman & Futter, 2008) can subsequently fuse with lysosomes to deliver their luminal contents for degradation (Klumperman & Raposo, 2014; Huber & Teis, 2016). In addition to proteolysis, MVBs and lysosomes carry out diverse cellular functions, including nutrient sensing (Sancak et al., 2007, 2008; Korolchuk et al., 2011), processing and loading of antigens for presentation (Kleijmeer et al., 2001) and exosome secretion for long-range communication between cells (Raposo & Stoorvogel, 2013). It is becoming increasingly clear that efficient and timely execution of their functions is intimately linked to the intracellular location and motility of late organelles (Neefjes et al., 2017); however, ways in which such attributes are regulated in space and time remain ambiguous. Here, we explore one aspect of this complexity by dissecting how changes in endolysosomal membrane identity are coupled to choice(s) of transport route.

To ensure productive interactions between diverse vesicles within the network and licence appropriate cargo flow, endosome identity must be clearly defined and easily interpreted. This identity, or maturation status, relies on progressive changes in the
endosome’s phospholipid repertoire (Schink et al., 2016), coupled to membrane occupancy by small GTPases that constitute the principal drivers of vesicular traffic (Wandinger-Ness & Zerial, 2014). Molecular switches of this type alternate between a GTP-bound state, corresponding to the active membrane-bound form, and a GDP-bound inactive state. Hence, their membrane residence time and biological activity are modulated by cognate guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs; Bos et al., 2007). For instance, while early endosomes typically carry the GTPase Rab5, late endosomes are instead marked by Rab7 (Zerial & McBride, 2001). During maturation, endosomes undergo a Rab5-to-Rab7 handover through an elegant mechanism, wherein Rab5 stimulates acquisition of Rab7 by recruiting its GEF, the Mon1/Ccz protein complex, while Rab7 in turn brings in the GAP for Rab5 (Nordmann et al., 2010; Poteryaev et al., 2010). This ordered transition serves as a gateway to the proteolytic compartment, establishing Rab7 as the central manager of transport and fusion events necessary for controlled delivery of endocytic, phagocytic and autophagic cargoes for degradation (Lanegmeyer et al., 2018). While, like Rab5, Rab7 can be removed from membranes through GAP-induced inactivation (Seaman et al., 2009; Carroll et al., 2013; Wong et al., 2018), whether it can also initiate a regulated handover to another GTPase is unknown.

Besides Rab7, late endosomes and lysosomes can also harbour Arl8b (Hofmann & Munro, 2006)—a GTPase implicated in diverse processes, including lysosomal degradation (Marwaha et al., 2017; Oka et al., 2017), antigen presentation and microbial killing (Garg et al., 2011), as well as nutrient sensing and autophagy (Jia et al., 2017; Pu et al., 2017). Arl8b is activated onto membranes by BORC, a multiprotein complex under the control of the mTOR pathway (Pu et al., 2015), and appears to coexist with Rab7 on a subset of late compartment structures (Mrakovic et al., 2012; Bento et al., 2013; Marwaha et al., 2017). By contrast to Rab7, which promotes perinuclear accumulation of the late compartment, acquisition of Arl8b is associated with positioning of vesicles in the periphery of the cell (Bonifacino & Neefjes, 2017). Due to the polar arrangement of microtubules, radiating from their juxtaplanar organizing centre (minus-end) towards the cell periphery (plus-end), movement into opposing directions involves at least two motor proteins—the dynein–dynactin complex for minus-end-directed transport, and one or more members of the kinesin family for transport to the plus-end (Granger et al., 2014). Desired motor complexes can be recruited to endosomal membranes on demand through appropriate GTPase effectors. Notably, Rab7 utilizes its effector RILP to recruit the dynein motor complex (Jordens et al., 2001), as well as associate with the Homo-typic fusion and Protein Sorting (HOPS) complex necessary for lysosomal degradation of materials contained within the MVB (van der Kant et al., 2015; McEwan et al., 2015a). On the other hand, Arl8b utilizes its effector SKIP/PLEKHM2 to recruit the kinesin-1 motor for transport towards the microtubule plus-end (Rosa-Ferreira & Munro, 2011). In addition, Arl8b can also attract the HOPS complex for fusion, either through SKIP (Khattar et al., 2015) or its family member PLEKHM1 (Marwaha et al., 2017). Recently, it was described that fusion between Rab7 and Arl8b vesicles gives rise to a hybrid identity compartment (Marwaha et al., 2017). However, what tactics the resulting endolysosomes employ to negotiate the choice between Rab7- versus Arl8b-directed transport routes remains unexplored. One option for ensuring spatiotemporal control under such conditions would be to simply remove one of the two GTPases, resulting in an autonomous single identity compartment.

To interrogate the above possibility, we studied ways in which established transport effectors of Rab7 and Arl8b influence the identity and location of late endosomes and lysosomes. We find that, while Rab7 and Arl8b can reside on the same membranes, expression of opposing transport route effectors RILP and SKIP spatially segregates the endolysosomal repertoire between morphologically distinct perinuclear and peripheral pools, respectively, marked by Rab7/RILP and Arl8b/SKIP. This segregation is predicated on an ordered GTPase switch from Rab7 to Arl8b, occurring on membranes destined for Arl8b/SKIP-mediated transport to the cell periphery. The switch involves recruitment of the GAP TBC1D15 to SKIP via its associated HOPS complex. Then, the GAP becomes competent to inactivate and remove Rab7 from SKIP-positive membranes—a situation analogous to Rab7-induced removal of Rab5 from hybrid Rab5/Rab7 endosomes. The greater endolysosomal system is thus controlled by consecutive handover mechanisms, with Rab5-to-Rab7 transition followed by a Rab7-to-Arl8b switch for regulated membrane transport.

**Results**

**Rab7 modulates the organization and dynamics of the Arl8b compartment**

To study transport route selectivity operational on endolysosomes, we examined the interplay between their associated GTPases, Rab7, and Arl8b. In agreement with previous findings (Marwaha et al., 2017), we noted that part of the repertoire populated by Rab7 also carries Arl8b (Fig 1A and B). It has been speculated that Arl8b marks a later maturation stage as compared to Rab7 (Hofmann & Munro, 2006; Garg et al., 2011), and we therefore considered whether Rab7 activity status affects localization and behaviour of Arl8b. In cells co-expressing wild type mCherry-Rab7, vesicular repertoire carrying Arl8b-GFP displayed bilateral organization in space and time (Fig 1C and D, and Movie EV1) characteristic of late compartments (Jongsma et al., 2016), with the bulk of vesicles congregated in the perinuclear (PN) region exhibiting less movement as compared to their peripheral (PP) counterparts. Expression of a constitutively active Rab7 mutant Q67L, which, due to its inability to complete the GTP hydrolysis cycle, cannot be released from membranes (Mukhopadhyay et al., 1997), resulted in loss of this PN/PP distinction, yielding disorganized movement of Arl8b-positive vesicles throughout the cell (Fig 1C and D). By contrast, expression of a dominant negative Rab7 mutant T22N, harbouring an inactive GTPase domain (Mukhopadhyay et al., 1997), rendered the cell periphery largely devoid of Arl8b-positive endolysosomes, causing a marked decrease in their motility throughout the cell (Fig 1C–F, and Movies EV2 and EV3). These results imply that Rab7 activity status impacts the localization and behaviour of organelles targeted by Arl8b.

**Rab7/RILP and Arl8b/SKIP complexes mediate spatial segregation of late endosomes**

To investigate the manner in which Arl8b and Rab7 coordinate spatial organization and motility of endolysosomes, we selectively
modulated specific transport routes through relevant effector molecules. Overexpression of the Rab7 effector RILP, responsible for recruitment of the dynein motor complex (Jordens et al., 2001), yielded acute perinuclear (PN) clustering of Rab7-positive endosomes (Fig 2A–C), rendering the cell periphery (PP) devoid of these structures. In the same cells, Arl8b-positive vesicles also exhibited PN accumulation in the presence of RILP (Fig 2A–C). On the other hand, introduction of the Arl8b effector SKIP for recruitment of plus end-directed motor kinesin-1 (Rosa-Ferreira & Munro, 2011) resulted in a concerted redistribution of Arl8b-positive endosomes to the periphery, with Rab7 also following suit (Fig 2A–C). This ability of Rab7 and Arl8b to respond to the other’s effector engagement suggested that the hybrid compartment is sensitive to demands exerted on both GTPases.

When both effectors were co-expressed in the same cells, segregation of Arl8b/SKIP from Rab7/RILP was observed
(Fig 2A–D), respectively, splitting the late compartment between PP and PN regions of the cell (Fig EV1A–C). Such segregation was not a result of mere activation of opposing transport machineries, as demonstrated by co-expression of RILP with FYCO1—an effector of Rab7 for plus-end-directed LE transport (Pankiv et al, 2010; Figs 2A–C and EV1A–C). Instead, when Rab7-positive endosomes were forced to move in both directions simultaneously, intermediate distribution of the late compartment was observed, illustrating a tug-of-war principle (Soppina et al, 2009; Hancock, 2014). Furthermore, engagement of Arl8b with
Plekhm1 instead of SKIP could not produce vesicle segregation in the presence of RILP (Figs 2A–C and EV1A–C), presumably due to lack of kinesin-interacting determinants in the resulting complex. Hence, spatial compartmentalization of the late endolysosomal repertoire is predicated on co-activation of opposing transport routes directed by different GTPases (Fig 2E), implicating RILP and SKIP in the maintenance of endolysosomal membrane identities.

To evaluate whether the PN/PP segregation observed upon co-activation of Rab7-driven transport to the microtubule minus-end and Arl8b-mediated plus-end-directed movement yields distinct subpopulations of organelles, we examined ultrastructural characteristics of affected compartments. To aid in detection of relevant organelles, HeLa cells, wherein CD63 was endogenously tagged with GFP, were used in combination with Sir-Lysosome staining (Fig 3). In unperturbed cells, analysis of the peripheral cytoplasm revealed predominantly MVBs harbouring homogeneous ILV contents, while later organelles, such as multilamellar lysosomes and endolysosomes exhibiting both multivesicular and multilamellar characteristics were more prevalent in the perinuclear region (Fig 3A). These observations are consistent with an earlier report demonstrating peripherally located late compartments to be less acidic and proteolytic relative to their perinuclear counterparts (Johnson et al., 2016).

Ultrastructural examination of the over-crowded PN region in cells ectopically expressing RILP revealed heterogeneous endolysosomal profiles, many of which appeared enlarged and harboured abnormal intraluminal contents (Fig 3B). Furthermore, neither canonical MVBs nor multilamellar lysosomes were appreciably observed in this condition (Fig 3B), suggesting that hyper-fusion between these morphologically distinct organelles may have taken place, giving rise to aberrant hybrid states. By contrast, in the presence of ectopically expressed SKIP, normal MVBs were preserved (Fig 3C). These organelles localized predominantly to the leading edge of vesicles migrating to cell tips in response to activation of plus-end-directed movement by SKIP (Fig 3C), implying that they constitute preferred targets for Arl8b/SKIP-mediated transport over their later counterparts (i.e. endolysosomes). Importantly, addition of SKIP in the background of RILP overexpression rescued normal MVB profiles, which, similar to the control situation, were located primarily in the cell periphery (Figs 3D and EV1D, Movies EV4 and EV5). These results indicate that a balance between the activities of Rab7 and Arl8b helps maintain a healthy MVB repertoire.

### SKIP is a negative effector of Rab7

Based on the evidence discussed above, we hypothesized that SKIP targets a subset of Rab7-positive membranes for transport to the cell periphery. Indeed, ultrastructural analysis confirmed that both proteins can occupy the same MVB membrane (Fig 4A) and co-isolate from cells (Fig 4B). Furthermore, the interaction between SKIP and Rab7 was strengthened by the constitutively active Rab7-Q67L (Fig 4B and C). This enhancement in binding was not observed for either RILP or Plekhm1 under the same reaction conditions (Figs 4B and EV1E), indicating that the interaction with SKIP is likely more transient. Importantly, binding of SKIP (like that of RILP and Plekhm1) was inhibited by Rab7-T22N (Figs 4B and C, and EV1E), indicating that SKIP prefers the active form of Rab7—a behaviour characteristic of an effector. We therefore tested whether the interaction between SKIP and Rab7 is direct. In vitro precipitation assays demonstrated that SKIP is able to bind recombinant GST-Rab7, albeit to a lesser extent than GST-Arl8b (Figs 4D and EV1F). Truncation analysis (Fig 4E) demonstrated that binding of SKIP to Rab7 does not involve the N-terminal RUN domain responsible for contacting Arl8b (Rosa-Ferreira & Munro, 2011). Instead, the C-terminal half of SKIP, spanning amino acids 537–1,019, mediates the interaction with Rab7, and removal of either residues 874–1,019 (construct 537–873) or 537–744 (construct 745–1,019) is detrimental to the interaction (Figs 4E and EV1H). Furthermore, alignment of the SKIP sequence against that of established Rab7 effectors revealed the presence of a canonical Rab7-interacting KML motif (McEwan et al., 2015b) at positions 610–612, with a conserved substitution of L for I (Fig 4E), and mutation of this KMI motif to AAA reduced the interaction between SKIP and Rab7 by roughly half (Fig 4F and G). Taken together, these findings demonstrate that SKIP binds Rab7 in a manner consistent with that of a bona fide effector and suggest that a single SKIP molecule could interact with both Rab7 and Arl8b (Fig 4H), thus setting the stage for a negotiation between them.

To investigate whether SKIP associates with Rab7-positive membranes prior to instigating plus-end-directed transport we designed a system wherein a pre-existing cytosolic pool of SKIP could be induced onto membranes. GFP-SKIP expression construct was fused to an oestrogen receptor (ER) fragment, allowing retention of the ER-GFP-SKIP chimera in the cytosol through an interaction with Hsp90 (Vigo et al., 1999; Rabinovich et al., 2008). Addition of tamoxifen would then release SKIP from Hsp90, recovering its intended localization and function, thereby bringing SKIP-mediated
transport under chemical control for timed release (Fig 4I). In the absence of tamoxifen, no stable targeting of ER-GFP-SKIP to endosomal membranes was observed (Fig 4J, Movie EV6). By contrast, within minutes following tamoxifen addition, ER-GFP-SKIP could be readily detected on mCherry-Rab7-positive vesicles, while no appreciable SKIP compartment had yet been generated at cell tips (Fig 4K). Over time, SKIP-positive vesicles increasingly accumulated at cell tips (the end points of plus-end-directed transport), while the population of Rab7-positive endosomes existing outside of the perinuclear cluster diminished (Fig 4K, Movies EV7 and EV8). These observations demonstrate that SKIP actively selects Rab7-positive membranes before initiating transport to the cell periphery.

Having shown that SKIP and RILP both target Rab7 and collaborate in segregation of late compartments between Rab7 and Arl8b, we tested whether loss of these effectors would result in the expansion of a hybrid compartment. Indeed, depletion of either RILP or SKIP augmented colocalization between endogenous Arl8b (tagged with GFP) and Rab7 (Fig 5A–C), complementing the overexpression studies presented in Fig 2. Given that SKIP interacts with both GTPases, but in the presence of RILP transports only Arl8b-positive endosomes to the cell periphery, we considered whether SKIP induces removal of Rab7 to ultimately achieve qualitative segregation of late compartments. To test this, we examined the effect of Rab7 activity on the identity of SKIP-positive vesicles. While ectopic expression of wild type GFP-Rab7 afforded efficient segregation between RILP and SKIP sub-compartments, expression of either constitutively active or inactive Rab7 mutants inhibited this process (Fig 5D and E). In the former perturbation, Rab7-Q67L and RILP

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Figure 3. Ultrastructural characterization of late compartment segregation along the perinuclear/peripheral axis.
A–D Correlative light and electron microscopy (CLEM) on (A) untransfected (WT) HeLa cells harbouring endogenous CD63 labelled with GFP as compared to those ectopically expressing (B) HA-RILP, (C) HA-SKIP or (D) both HA-RILP and HA-SKIP. Left panels: wide-field fluorescence images of fixed cells showing endogenous GFP-CD63 (green) and SiR-lysosome-stained endosomes and/or lysosomes (red). Cell and nuclear boundaries are demarcated with solid and dashed lines, respectively, and zoom insets highlight regions selected for EM imaging. Middle panels: overview electron micrographs of perinuclear (PN) and peripheral (PP) cell regions selected for further analysis. Various endolysosomal subtypes are designated by arrowheads: MVBs (white), lysosomes (black), endolysosomes (yellow) and abnormal/enlarged endolysosomes (red). Right panels: graphical representations of endosomal distribution under the indicated conditions based on ultrastructural characterization. Scale bars as indicated (see also Fig EV3D, and Movies EV4 and EV5).
Figure 4.
were retained on peripheral SKIP-positive vesicles, while in the latter case, due to the dominant negative quality of Rab7-T22N, the Rab7/RILP compartment was absent, and no segregation between RILP and SKIP could be observed (Fig 5D and E). These data implicate GTPase activity status of Rab7 in the compartmentalization of the endosomal repertoire and suggest that inactivation and removal of Rab7 from the endosomal membrane takes place with commencement of SKIP-associated transport (Fig SF).

**GAP TBC1D15 removes Rab7 from membranes designated by SKIP**

Inactivation of Rab GTPases is typically facilitated by cognate GAP molecules in possession of Tre-2/Bub2/Cdc16 (TBC) domains (Fukuda, 2011). We hypothesized that loss of relevant GAP activity would maintain active Rab7 on structures selected by Arl8b/SKIP, thus giving rise to a tug-of-war phenotype characterized by breakdown of bilateral architecture and concomitant disorganization of transport. Three TBC family proteins previously described to possess GAP activity towards Rab7 were examined (Fig 6A): TBC1D2, implicated in ILV formation and (Carroll et al, 2013; Jaber et al, 2016); TBC1D5, associated with the retromer complex (Jimenez-Orgaz et al, 2018; Seaman et al, 2018); and TBC1D15, described to operate at endosome-mitochondria contact sites (Zhang et al, 2005). Depletion of TBC1D2 did not markedly affect intracellular distribution of the late compartments carrying CD63, while knockdown of either TBC1D5 or TBC1D15 perturbed the PP/PN balance (Fig 6B and C). The former scenario led to accumulation of CD63-positive structures at cell tips, reflecting an exaggerated PN/PD divide. By contrast, loss of TBC1D15 produced dispersion of late compartments (Figs 6B and C, and EV2A and B) throughout the cell compartments (Figs 6B and C, and EV2A and B) throughout the cell.

By contrast, loss of TBC1D15 produced dispersion of late compartments carrying CD63, while in control cells nearly all membrane-localized Rab7 remained in PN clusters marked by RILP (Figs 7A–D and EV2A–C). Notably, co-transport of RILP with endogenous Rab7, retained on peripheral structures marked by SKIP, was not observed under conditions of TBC1D15 loss (Fig 7A), remaining on perinuclear Rab7 instead. This observation echoes a protective phenomenon previously reported for other effector proteins when bound to their client GTPase (Nagelkerken et al, 2000) and imply that SKIP-associated TBC1D15

considerations, we concluded that of the 3 known Rab7 GAPs, TBC1D15 is most likely to play a direct role at the Rab7/Arl8b interface, while TBC1D5 regulates a parallel pathway emanating from the same organelles.

To integrate TBC1D15 function within the cell biological context of Arl8b/Rab7 interplay, we tested the consequences of TBC1D15 insufficiency on organelle architecture and motility of late compartments. Ultrastructural examination revealed loss of normal MVB and lysosome morphologies in cells depleted of TBC1D15. Instead, late organelles were now comprised of enlarged endolysosomes harbouring aberrant luminal contents (Fig 6F), closely resembling alterations incurred upon overexpression of RILP (Fig 3B). Additionally, loss of TBC1D15 gave rise to disorganized movement of endolysosomes in cellular space (Fig 6G, and Movies EV9 and EV10), akin to that observed in cells expressing constitutively active Rab7 Q67L (Fig 1C). This was accompanied by a marked reduction in the fast-moving vesicle repertoire (Fig 6H), pointing to a pivotal role for TBC1D15 in the regulation of peripheral LE/Ly motility. In agreement with a previous report (Peralta et al, 2010), depletion of TBC1D15 reduced cycling of Rab7 on endosomal membranes (Fig 6I), indicating altered activity dynamics.

We next tested whether TBC1D15 influences partitioning of the endolysosomal pool between the PN cloud and the cell periphery upon co-activation of opposing transport routes downstream of Rab7 and Arl8b. Silencing TBC1D15, but not its homologue TBC1D17, led to redistribution of a substantial proportion of endogenous Rab7 pool to the tips of cells (PP) along with SKIP, while in control cells nearly all membrane-localized Rab7 remained in PN clusters marked by RILP (Figs 7A–D and EV2A–C). Notably, co-transport of RILP with endogenous Rab7, retained on peripheral structures marked by SKIP, was not observed under conditions of TBC1D15 loss (Fig 7A), remaining on perinuclear Rab7 instead. This observation echoes a protective phenomenon previously reported for other effector proteins when bound to their client GTPase (Nagelkerken et al, 2000) and imply that SKIP-associated TBC1D15
Figure 5.
preferentially targets Rab7 not in complex with RILP. As before, depletion of TBC1D12 showed little effect in this context, while loss of TBC1D15 exhibited a similar phenotype to that of TBC1D15 (Figs 7B–D and EV2D), suggesting that the retromer/TBC1D5 pathway may compete with SKIP/TBC1D15 for the same Rab7 substrate. To ensure that the phenotypes observed above were not due to inactivation of another potentially relevant GTPase by TBC1D15, we conducted in vitro assays confirming that TBC1D15 specifically accelerates GTP hydrolysis of the GTPase domain belonging to Rab7, without affecting those of Rab5, Ran or Rab9 (Fig EV2E). Furthermore, Rab7 cargo MHC class II (MHC-II) was found to aberrantly traffic together with SKIP the absence of TBC1D15 (Figs 7E and EV2F), underscoring the notion that membrane dynamics at the Rab7 endosome are modulated at least in part through the interplay of SKIP and TBC1D15.

**SKIP recruits TBC1D15 not associated with mitochondria**

Having shown that depletion of TBC1D15 hampers removal of Rab7 from the peripheral SKIP compartment, we tested whether SKIP is able to target this GAP to its membranes of choice. TBC1D15 localizes predominantly to the outer mitochondrial membrane (Onoue et al., 2013; Yamano et al., 2014) and has previously been shown to target Rab7 at membrane contact sites between mitochondria and endosomes (Wong et al., 2018). However, FRAP analysis performed on endogenous TBC1D15, N-terminally tagged with GFP in HeLa cells (Fig EV3A), revealed its association with mitochondrial membranes to be dynamic (Fig EV3B). Additionally, TBC1D15 negative for mitochondrial markers was found to colocalize with Rab7- and CD63-positive endosomes in unperturbed cells, as well as under depletion of the mitochondrial TBC1D15 anchor, FIS1 (Fig 8A and B), implying that TBC1D15 can be delivered to endosomes by other mechanisms. In support of this, FIS1 knockdown did not recapitulate key phenotypes of TBC1D15 loss of function, affecting neither the intracellular distribution of CD63-positive structures nor retention of Rab7 on the SKIP compartment (Figs 8C–F and EV3C).

Upon ectopic expression of SKIP, endogenous TBC1D15 could be detected at SKIP-positive vesicles located in the tips of cells, which did not co-label with either Tomm20 or MitoTracker (Figs 8G–J, and EV3D and E). On the other hand, TBC1D15 did not show appreciable colocalization with PLEKH1M (Fig 8G and H), indicating specificity in recruitment of this GAP to endosomal membranes. Taken together, these results demonstrate that TBC1D15 can be recruited to membranes selected by SKIP and imply that this acquisition does not involve direct contacts with mitochondrial (Fig 8K).

**TBC1D15 interacts with the HOPS complex to inactivate Rab7 from SKIP-positive membranes**

To understand the molecular underpinnings of TBC1D15 recruitment to SKIP for inactivation of Rab7, we first sought to place SKIP/Rab7 interactions in the context of the greater Arl8b/SKIP transport complex. Both Arl8b and SKIP have been shown to recruit the HOPS complex to late endosomes by directly contacting VPS41 and VPS39 subunits, respectively (Khattar et al., 2015). Co-precipitation analysis of SKIP truncations mapped the interaction with VPS39 onto the central region of SKIP, overlapping the KMI motif, while its association with VPS41 extended further towards SKIP’s N-terminus (Fig EV4A and B). This in turn suggested that engagement of Rab7 may influence acquisition of the HOPS complex by SKIP. Indeed, SKIP-AAA exhibited strongly reduced affinity for VPS39, as compared to its wild-type counterpart (Fig 9A and B), resulting in failure to recruit VPS39 to endosomes (Fig 9C and D). At the same time, co-isolation with VPS41 and its recruitment were improved by SKIP-AAA (Figs 9A–C and EV4C). Importantly, the SKIP mutant lacking the RUN domain failed to localize to endosomes (Figs 9C and D, and EV4C), indicating that association with Arl8b is a prerequisite for SKIP function on these organelles. Taken together, these results suggest that SKIP-associated HOPS assembly relies on contacts to both of its cognate GTPases.

Strikingly, co-expression of VPS39 with SKIP caused a marked improvement in acquisition of endogenous TBC1D15 (Fig 9E and F), and similar effects were observed for ectopic expression of other HOPS subunits (Figs 9E–G and EV4D). Furthermore, in all cases, co-recruitment of endogenous core HOPS subunits VPS39 and VPS51 was also observed (Fig 9H and I), implying that the complex (rather than isolated subunits) is involved in recruitment of TBC1D15 to SKIP-positive membranes. As expected, no contribution from FIS1 was observed in this context (Figs 9G and EV5A), excluding direct contribution of mitochondria-bound TBC1D15.

We next examined whether TBC1D15 is a component of the SKIP-associated complex. Because only a faint interaction was detected between SKIP and TBC1D15 by conventional co-IP (Fig EV5B), we turned to the BioID approach (Roux et al., 2012).
allowing interactors of a protein of interest to be covalently labelled with biotin in cells prior to lysis. SKIP was adapted with the BioID variant of a promiscuous biotin ligase domain, and in the presence of exogenously supplied biotin, TBC1D15 was biotinylated by BioID-SKIP at levels far exceeding those afforded by the free BioID moiety under the same conditions (Fig 10A and B). In the same experiment, specific labelling of endogenous VPS18 by BioID-SKIP was also observed, indicating that both VPS18 and TBC1D15 are in complex with SKIP. Similarly, endogenous VPS18 (and VPS11) were detected at the SKIP compartment together with TBC1D15 (Fig EV5C). Taking these results together with enhanced recruitment of TBC1D15 resulting from overabundance of HOPS subunits
A-C Identification of a Rab7 GAP that promotes endolysosomal system’s bilateral architecture along the PN/PP axis. (A) Graphical summary depicting the functions of 3 known TBC domain-containing GAPs for Rab7. (B) Representative confocal images of fixed HeLa cells depleted of TBC1D2, 1D5 or 1D15 using siRNA oligo pools and immunolabelled against CD63 (white), scale bar: 10 μm. (C) Plot of CD63 pixel distribution as a function of TBC1D2/1D5/1D15 depletion expressed as fractional distance along a straight line from centre of nucleus (0) to the plasma membrane (1.0), number of (pixels) plotted given above each scatter, n ≥ 8 cells per condition analysed from 2 independent experiments. Significance: one-way ANOVA test (relative to siC), ***P < 0.001 (see also Fig EV2A and B).

D-E Rescue of TBC1D15 depletion phenotype. (D) Representative confocal images of fixed HeLa cells transfected with either control siRNA (siC) or oligo #3 targeting TBC1D15 (siTBC1D15) and ectopically expressing either GFP-EV, GFP-TBC1D5 or siRNA-resistant GFP-TBC1D15res (green), immunolabelled for CD63 (magenta), scale bar: 10 μm. (E) Quantification of rescue expressed as % cells (average) in the population exhibiting one of 3 phenotypes: clustered, dispersed or intermediate; total numbers of cells analysed per condition appear above each bar, n = 3 independent experiments. Significance (based on clustered phenotype): one-way ANOVA test relative to either siC/EV (top row) or siD15/EV (bottom row), ***P < 0.001, ns: not significant.

F-H Effects of TBC1D15 depletion on the morphology and dynamics of late organelles. (F) Representative electron micrographs of fixed HeLa cells transfected with either control siRNA (siC) or a pool of oligos targeting TBC1D15 (siTBC1D15) are shown, scale bars as indicated. (G) Analysis of late compartment dynamics as a function of TBC1D15 depletion. Tracks followed by Sir lysosome-positive vesicles during a time-lapse lasting 255 s (5 s per frame), with highest displacement rates for each track depicted on a rainbow colour scale (blue: immobile; red: maximum mobility per time interval). Zoom insets (3×) highlight select peripheral (PP) and perinuclear (PN) regions, scale bar: 10 μm (also see Movies EV9 and EV10). (H) Quantification of high motility fraction (% tracks with displacement rates above 0.9 μm/s), ntr = 27, ntr-D15 = 37 images (2 ± cells per image) analysed from 2 independent experiments. Effectiveness of TBC1D15 (siD15) depletion is confirmed by immunoblot (also appearing in Fig EV2A) against endogenous TBC1D15 (eD15) following its immunoprecipitation (IP). TL: total lysate.

I FRAP of GFP-Rab7 in live HeLa cells transfected with either control siRNA (siC, black line) or a pool of oligos targeting TBC1D15 (siD15, red line). Plotted is average GFP-Rab7 signal recovery during 200 s following bleaching, expressed as % of pre-bleach signal, n = 3 bleach regions per sample. Data information: Cell and nuclear boundaries are demarcated with solid and dashed lines, respectively, as applicable. Graphs report mean (red line) of sample values (open circles), and error bars reflect ± SD. Unless stated otherwise, significance was assessed using two-tailed Student’s t-test, ***P < 0.001.

Source data are available online for this figure.

Discussion

Maturation and motility of endosomes are coordinated by their ever-changing membrane-associated protein interactomes that mark different endosomal states. In particular, controlled arrivals and departures of GTPases to and from specific endosomal membranes determine the duration of time those endosomes persist in a given maturation state (temporal regulation) and inform their transport itineraries (spatial regulation). Most immediately, residence of specific GTPases on endosomal membranes is modulated by their cognate GEFs and GAPs, respectively, turning relevant GTPase activities “on” and “off”. As such, control over the GTP hydrolysis cycle is instrumental in achieving regulated transitions from one GTPase to another, thereby timing endosomal maturation and controlling GTPase-directed transport. Until now, this type of GTPase handover mechanism on endosomes has been most extensively described for the Rab5-to-Rab7 conversion taking place during the critical early-to-late maturation step (Rana et al., 2015).

In this study, we propose an additional handover mechanism occurring at the late endosomal stage—the Rab7-to-Ar18b switch. In this stepwise transfer, the GAP necessary to inactivate and remove Rab7 from mature MVBs is provided by the Ar18b/SKIP complex, thus inducing controlled disengagement of Rab7.

Because both Rab7 and Ar18b can mediate transport of vesicles towards the periphery, a question arises as to why the same endosomal compartment allows for two plus-end-directed transport machineries. Our observations suggest that installation of opposing transport machineries on different GTPases allows not only spatial but also qualitative (i.e. morphological) control over the LE/Ly repertoire. We characterize SKIP as a dual specificity effector, engaging Rab7 as well as Ar18b, which sets up a platform for a dialogue between them. This parallels what has recently been described for SKIP’s family member PLEKHM1 (Marwaha et al., 2017). However, while PLEKHM1 bridged Ar18b and Rab7 residing on opposing membranes during fusion, resulting in a hybrid identity
A–D Effects of depleting known GAPs for Rab7 on late compartment segregation mediated by RILP and SKIP. (A) Representative confocal images of fixed HeLa cells transfected with either control siRNA (siC), two different siRNA oligos targeting TBC1D15 (#1 and #3) or oligo pool targeting TBC1D17 and ectopically expressing GFP-Arl8b (green) in combination with HA-RILP (cyan) and Myc-SKIP (red), immunolabelled against endogenous Rab7 (eRab7, magenta) and the indicated epitope tags. Cell and nuclear boundaries are demarcated with solid and dashed lines, respectively, and zoom insets (in response to depletion of TBC-containing proteins, (PN) cell regions, scale bar: 10 μm. Graphical summaries appear on the right of each condition. (B) Colocalization of SKIP with endogenous Rab7. (C) Plot of Rab7-positive pixel distribution expressed as fractional distance along a straight line from centre of nucleus (0) to the plasma membrane (1.0), number of (pixels) plotted given above each scatter, n = 8 cells analysed per condition from 2 independent experiments. (D) Validation of depletion efficiencies expressed as fraction of the indicated mRNA remaining relative to siC assayed by qPCR, n = 8 cells analysed from 2 independent experiments. (E) Effect of TBC1D15 depletion on Rab7-dependent MHC-II receptor trafficking to the SKIP-positive compartment, n_AIC = 21, n_AIC15 = 21 cells analysed from 3 independent experiments (see also Fig EV2F). Significance: two-tailed Student’s t-test, ***P < 0.001.

Data information: Graphs report the mean (red line) of sample values (open circles), and error bars reflect ± SD. Unless stated otherwise, significance was assessed using one-way ANOVA test (relative to siC). *P < 0.05, **P < 0.01, ***P < 0.001, ns: not significant.
Figure 8.
compartment, SKIP likely engages these GTPases on the same membrane. The latter scenario ultimately leads to the removal of Rab7 from target membranes, restoring the Arl8b single identity repertoire. Delving deeper into the mechanism, we find that SKIP harbours a variant of the KML motif conserved in other Rab7 effectors and utilized to make direct contacts with the GTPase. In the case of SKIP, L is substituted by I, which may be responsible for the apparent lower affinity of Rab7 for this effector. This would be consistent with the proposed negative, and hence transient, effector function of SKIP towards Rab7 benefiting from easy disengagement. Curiously, the sequence context surrounding KMI residues in SKIP is closer to that of FYCO1, rather than for instance PLEKHM1 (McEwan et al., 2015b), suggesting that SKIP and FYCO1 may have evolved to compete for the same Rab7 substrate. What then determines which plus-end-directed pathway is chosen—and why—remains an open question. One obvious possibility is that the presence of Arl8b in close proximity to Rab7 skews the system towards SKIP, while its absence endows FYCO1 with the default plus-end effector status. Likewise, it is conceivable that formation of ER-endosome contacts organized by protrudin (Raiborg et al., 2015) would instead be promotive of the FYCO1-dependent transport route. How these and other mechanisms impinging upon transport route selectivity by late endosomes are integrated to support orderly spatiotemporal regulation of these complex organelles remains to be investigated.

For the Rab7-to-Arl8b switch to commence, Arl8b needs to either be activated and recruited to Rab7-positive structures, or already be found on the same membrane due to a prior fusion event (Marwaha et al., 2017). Subsequently, Arl8b-associated molecular machinery must come in contact with Rab7 to deliver the GAP for its inactivation and release. The first action is known to be mediated by the BORC complex, which is reported to function as a GEF for Arl8b (Pu et al., 2015). Interestingly, BORC shares a number of subunits with BLOC1, the complex reported to induce removal of Rab5 through the recruitment of the cognate GAP Msh5. Overlap in subunit build-up is not uncommon in remodelling of membrane identity, as illustrated by the VPS core shared between the CORVET and HOPS tethering complexes operating on early and late endosomes, respectively (Balderhaar & Ungermann, 2013). Because the HOPS complex can be recruited by both Rab7 and Arl8b (van der Kant et al., 2015; Khatter et al., 2015), and activation of Arl8b by BORC has been shown to promote acquisition of HOPS by autolysosomes (Jia et al., 2017), HOPS constitutes a common factor ideally positioned to facilitate an ordered transition from Rab7 to Arl8b. Indeed, we find that the interaction between SKIP and Rab7, established through the KMI motif, supports acquisition of VPS39 by the SKIP-associated HOPS. Interestingly, the yeast homologue of VPS39 functions as a GEF for the Rab7 homologue Ypt7, but this attribute is not conserved in mammals (Peralta et al., 2010). Our observations suggest that in higher organisms this HOPS subunit has taken on the role of a sensor of Rab7 in macromolecule complexes assembled on endosomal membranes. Additionally, we find that in the absence of Rab7 contacts, SKIP exhibits higher affinity for other HOPS subunits, including VPS41 provided by Arl8b (Khatter et al., 2015), suggesting that SKIP and the associated HOPS complex straddle the two GTPases, poised for timely delivery of the Rab7 GAP.

In this study, we implicate the GAP TBCD1D15 in the second step of the Rab7-to-Arl8b conversion—the inactivation and release of Rab7 from endosomal membranes selected by SKIP. TBCD1D15 is not the only described GAP with biochemical activity against the Rab7 GTPase (Seaman et al., 2009; Frasa et al., 2010), and GAP molecules in general are known to operate with a certain degree of promiscuity (Fukuda, 2011). Therefore, GAP specificity is unlikely to rely exclusively on its catalytic activity, but rather depends on controlled targeting of said activity to the correct location at the appropriate time. For instance, TBCD1D2A (Armus) has been shown to inactivate Rab7 during lysosomal maturation and starvation-induced autophagy (Carroll et al., 2013; Jaber et al., 2016), while TBCD1D5 functions with the retromer complex to remove Rab7 participating in endosome-to-Golgi transport as well as in early steps of autophagy (Seaman et al., 2009; Popovic & Dikic, 2014). Our findings suggest that TBCD1D5, but not TBCD1D2, may compete with the SKIP/TBCD1D15 pathway for the same Rab7 substrate, as loss of this GAP produces a phenotype akin to hyper-segregation of late compartments observed with overabundance of SKIP and RILP. In addition to other Rab7 GAPS, TBCD1D15 itself targets Rab7 at different cellular locations for distinct biological purposes. Endogenous TBCD1D15 localizes primarily to mitochondrial membranes, where it participates in mitophagy and ER-curated mitochondrial fission (Onoue et al., 2013; Yamano et al., 2014; Wong et al., 2018). We now find that mitochondrial
TBC1D15 appears to coexist in equilibrium with its cytосolic pool, and that Arl8b/SKIP/HOPS draws from the latter to conduct plus-end-directed transport of MVBs into the cell periphery. Such coupling of membrane identity to directed motility echoes the famed Rab5-to-Rab7 conversion taking place earlier in the lifespan of an endosome when maturation is linked to transport towards the perinuclear proteolytic hub.

Spatiotemporal control of GTPase association with their target membranes bears consequences for transport of membranes and cargoes towards and away from relevant sub-compartments within the endolysosomal system. As the Rab5-to-Rab7 handover mechanism marks the timing of early-to-late transitions along the endocytic route, coupled to MVB formation and transport of these structures towards the cell’s interior, the Rab7-to-Arl8b conversion described here defines a further membrane identity switch coupled to movement of select organelles back into the periphery. Arl8b has
Figure 10.
been described to target later organellar profiles as compared to Rab7 (Hofmann & Munro, 2006; Garg et al., 2011). On the one hand, our description of SKIP-dependent ejection of Rab7 to form a temporarily later Arl8b-positive single identity compartment support this order of events. Conversely, ultrastructural evaluation indicates that Rab7 together with RILP preside over morphologically “later” organelles, as compared to the peripheral MVBs targeted by Arl8b and SKIP in the same cells. Taken together with recent work by others (Johnson et al., 2016), our observations argue that the definition of what constitutes mature and/or late organelles within the cell’s heterogeneous acidic and proteolytic repertoire is ever more complex and intriguing. Our findings further support the emerging paradigm of Rab7 as a central regulatory node in the endolysosomal system, capable of a wide range of partnerships necessary to manage the diverse lifestyles of acidic and proteolytic compartments.

Materials and Methods

Cell lines and culturing

HEK293T (human embryonic kidney) and HeLa (human cervical carcinoma) cell lines were purchased from ATCC and cultured in DMEM (Gibco) supplemented with 7.5% FCS. MelJuSo (human melanoma) cells were kindly provided by G. Riethmuller (LMU, Munich) and cultured in IMDM (Gibco) supplemented with 7.5% foetal calf serum (FCS, Greiner). HeLa cells harbouring endogenous CD63, Arl8b, or TBC1D15 N-terminally tagged with GFP were generated using CRISPR/Cas9. To create an endogenous GFP-tag vector, the CMV promotor from mGFP-C1 was replaced for MCS (linker) vector. GST-Ran has been previously described (Izaurralde et al., 1997). mCherry-Rab7 [G. Voeltz, Addgene plasmid #61804 (Rowland et al., 2014)] was used to generate mCherry-Rab7 Q67L and T22N mutants by site-directed mutagenesis using standard protocols. Arl8b-GFP was a gift from S. Munro (Hofmann & Munro, 2006) and was used to generate Myc-Arl8b by cloning it into the 2xMyc-C1 vector at KpnI/XhoI restriction sites from canine Rab7a cDNA (NM_001003317.2) was cloned into pRP-261 bacterial expression vector. Human SKIP cDNA (Rosa-Ferreira & Munro, 2011) was cloned into mRFP-C1 (BglII/HindIII) and 2xMyc-C1 (BglII/EcoRI) vectors, respectively, using a ligation-independent cloning approach (Luna-Vargas et al., 2011). Canine Rab5 cDNA (coding for aa 1–210, NM_001003317.2) was cloned into pREP-261 bacterial expression vector BamHI/EcoRI restriction sites. Human Rab9a cDNA (NM_004251) was cloned into pET15B-His bacterial expression vector at KpnI/BamHI restriction sites from canine Rab7a cDNA (NM_001003316.1) and murine Arl8b cDNA (NM_026011.3), respectively, using a ligation-independent cloning approach (Luna-Vargas et al., 2011). Canine Rab5 cDNA (coding for aa 1–210, NM_001003317.2) was cloned into pREP-261 bacterial expression vector BamHI/EcoRI restriction sites. Human Rab9a cDNA (NM_004251) was cloned into pET15B-His bacterial expression vector. GST-Ran has been previously described (Izaurralde et al., 1997).

DNA constructs

GFP-Rab7 and Myc-Rab7 have been described before (Jordens et al., 2001), and GFP-Rab7 Q67L and T22N were gifts from P. Chavrier (Meresse et al., 1995). mcherry-Rab7 [G. Voeltz, Addgene plasmid #61804 (Rowland et al., 2014)] was used to generate mcherry-Rab7 Q67L and T22N mutants by site-directed mutagenesis using standard protocols. Arl8b-GFP was a gift from S. Munro (Hofmann & Munro, 2006) and was used to generate Myc-Arl8b by cloning it into the 2xMyc-C1 vector (KpnI/BamHI restriction sites). GST-Rab7 and Myc-Rab7 have been described before (Jordens et al., 2001), and GFP-Rab7 Q67L and T22N were gifts from P. Chavrier (Meresse et al., 1995). mcherry-Rab7 [G. Voeltz, Addgene plasmid #61804 (Rowland et al., 2014)] was used to generate mcherry-Rab7 Q67L and T22N mutants by site-directed mutagenesis using standard protocols.

| Primer name | Sequence |
|-------------|----------|
| CD63 Endo BglIII FW | CCCAGATCTATGGCGGTGAGGGGAGGAAT |
| CD63 Endo EcoRI RV | CCCAGAATTCAGAGAATTACGACGAGGA |
| CD63 gRNA FW | CACGGCGCGGCGACATGGCGGAGGA |
| CD63 gRNA RV | AAATCCCGCGCATTGCGCCG |
| Rab7 GAP nEndo Ndel FW | CCCCAACATGGTCACAGACGTAATTAAATGTC |
| Rab7 GAP nEndo PinAI RV | CCCAAGCTTCGGTGAGTTTGAG |
| Rab7 GAP nEndo BglIII FW | CCCAGATCTCGCGGTGACGGGTGAGGA |
| Rab7 GAP nEndo EcoRI RV | CCCAGAATTCACGGCGAGTTAAATAAGCTTA |
| Rab7 gRNA FW | CACGGCGCGGCGACATGGCGGAGGA |
| Rab7 gRNA RV | AAATCCCGCGCATTGCGCCG |
| ARL8b nEndo BglIII FW | ACTACCAGATCTTCTGGCGCTACTTCAGC |
| ARL8b nEndo EcoRI RV | ACTACCAGAATTCACGGCGAGTTAAATAAGCTTA |
| ARL8b nEndo Ndel FW | ACTACCAGATCTTCTGGCGCTACTTCAGC |
| ARL8b nEndo EcoRI RV | ACTACCAGAATTCACGGCGAGTTAAATAAGCTTA |
| ARL8b gRNA FW | CACGGCGCGGCGACATGGCGGAGGA |
| ARL8b gRNA RV | AAATCCCGCGCATTGCGCCG |
| ARL8b gRNA RV | AAATCCCGCGCATTGCGCCG |

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2004). SKIP was then cloned into the resulting vector (BglII/NotI restriction sites). HA-TurboID-SKIP and the corresponding vector control were generated as follows. 2xHA-TurboID fragment was amplified from 3xHA-TurboID-NLS-pcDNA3 (A. Ting Addgene plasmid #107171) and cloned into mGFP-C1 vector (Nhel/BglII restriction sites) to substitute GFP, resulting in 2xHA-TurboID-C1. Subsequently, SKIP was cloned into 2xHA-TurboID-C1 at BglII/ EcoRI restriction sites.

Myc-SKIP truncations and RFP-SKIP KMI mutants were generated using In Vivo Assembly (IVA) method (Garcia-Nafria et al., 2016). In short, a reaction mix containing 0.2 μM forward and 0.2 μM reverse primer (18 nucleotides specifically designed), 200 μM dNTPs, 0.25 ng template, 1× Phusion HF Buffer, 0.25 μl Phusion DNA Polymerase, and filled to 25 μl with MilliQ was amplified using the following programme 98°C 3 min; (98°C 10 s; 60°C 30 s; 72°C 15 s/Κb) × 25 cycles; 72°C 5 min; 4°C forever. Reaction products were digested with 1 μl Dpni for 15 min at 37°C and 2 μl subsequently transformed into the Escherichia coli DH5α strain. All constructs used in the study were sequence verified.

Human RILP CDNA (Jordens et al., 2001) was cloned into mRFP-C1 and HA-C1 vectors using BglII and HindIII restriction enzymes, PLEKH1M-FLAG (Wijdeven et al., 2016). GFP-FYC01 was a gift from T. Johansen (Pankiv et al., 2010).

Murine His-TBC1D15, a gift from X. Zhang (Zhang et al., 2005), was used to clone TBC1D15 into mGFP-C1 and mRFP-C1 (NcoI/SpeI restriction sites). GFP-TBC1D15 was then used to create the inactive mutant R417A and siRNA-resistant mutants by site-directed mutagenesis according to standard protocols. GFP-TBC1D5 was a gift from M. Seaman (Seaman et al., 2009).

HOPS complex constructs for mCherry-VPS11, mCherry-VPS16N, mCherry-VPS18, RFP-VPS33B, RFP-VPS39 and mCherry-VPS41 have been previously described (van der Kant et al., 2015).

DNA and siRNA transfections

DNA transfections of HeLa and MelJuSo cells were carried out using Effectene (Qiagen #304127), according to manufacturer's protocols. HEK293T cells were transfected using Polyethylenimine (PEI) (Polyfectene (Qiagen #301427), according to manufacturer's protocols. DNA transfections of HeLa and MelJuSo cells were carried out using Polyethylenimine (PEI) (Polyfectene (Qiagen #301427), according to manufacturer's protocols. DNA and siRNA transfections mCherry-VPS18, RFP-VPS33B, RFP-VPS39 and mCherry-VPS41 have been previously described (van der Kant et al., 2015).

Antibodies, reagents and fluorescent dyes

Confoal microscopy

The following primary antibodies were used for immunofluorescence: rabbit anti-Rab7 (Cell Signalling #D95F2), anti-MHC-II (Neefjes et al., 1990), anti-VPS11 (Abcam #ab125083), anti-VPS18 (Abcam #ab178416) and anti-TBC1D15 (Sigma # HPA013888), as well as mouse anti-CD63 NKI-C3 (Vennekoor & Rumke, 1986), anti-LAMP1 (H4A3, Santa Cruz), anti-TOM20 (Abcam #ab56783), anti-Myc (9E10, Sigma) and anti-FLAG M2 (Sigma #1804), as well as rat anti-HA (3F10, Roche). Secondary donkey anti-rabbit/anti-mouse (Invitrogen) or anti-rat (Biotium) Alexa-coupled antibodies were subsequently used for fluorescence detection, as applicable. Mitotracker Red CMXRos (Invitrogen) and SiR-Lysosome (Spirochrome #CY-SC012) were used to visualize mitochondria (100 nM, for 30 min) and late endosomes/lysosomes (2 μM, for 30–60 min), respectively. 4-OH-Tamoxifen (used at 0.1 μM final concentration) was a gift from W. Zwart (NKI).

Western blotting

The following primary antibodies were used for detection by immuno: blot: rabbit anti-mGFP (Rocha et al., 2009), anti-mRFP (Rocha et al., 2009) and anti-VPS18 (Abcam #ab178416), as well as mouse anti-RFP (5F8, Chromotek), anti-HA (HA.11 (16B12), Covance), anti-Myc (9E10, Sigma), anti-GST (2F3) (van Zeijl et al., 2007), anti-β-actin (AC-15, Sigma), anti-FLAG M2-PO (Sigma #AB592) and anti-HA PO (Roche #1203819001). The following secondary detection agents were used when applicable: rabbit anti-mouse-PO (Dako #P0161), HRP-Protein A (Invitrogen #10-1023), IRDye 800CW goat anti-rabbit IgG (H+L) (LI-COR #926-32211), IRDye 800CW goat anti-
mouse IgG (H+L) (Li-COR #926-32210), IRDye 680LT goat anti-rabbit IgG (H+L) (Li-COR #926-68021) and IRDye 680LT goat anti-mouse IgG (H+L) (Li-COR #926-68020).

**Immunoprecipitation**

Rabbit anti-TBC1D15 (Abcam #ab121396) was used to isolate TBC1D15 from lysates, as well as for subsequent immunoblotting.

**Immunogold EM**

Biotinylated goat anti-GFP (#600-106-215) and anti-biotin (#100-4198) were both obtained from Rockland Immunochemicals, and anti-RFP (#R10367) was purchased from Molecular Probes.

**Fluorescence microscopy**

Fixed sample preparation for confocal microscopy was performed according to standard protocols. Samples were mounted using ProLong Gold antifade (P10144, Invitrogen) and imaged on Leica SP8 microscope equipped with appropriate filters for fluorescence detection. Images were acquired using a Hcx PL 63 × 1.32 oil objective and 1–4 digital zoom as applicable. Alexa-405 was excited at λ = 405 nm and detected at λ = 416–470 nm; Alexa-488 was excited at λ = 488 nm and detected at λ = 500–550 nm; Alexa-568 was excited at λ = 561 nm and detected at λ = 570–621 nm; Alexa-647 was excited at λ = 633 nm and detected at λ = 642–742 nm. Pixel plot graphs were created by using the line profile tool of LAS-AF. Colocalization was reported as Mander’s overlap calculated using JACoP plug-in for ImageJ (displayed as protein A: protein B). For live cell imaging, cells were grown on 35-mm glass bottom dishes (1.5 coverglass, MatTek) coated with poly-d-lysine. Cells were treated as applicable, and time-lapses were collected on the Dragonfly spinning disc microscope or Leica WLL microscope, both adapted with climate control chambers. Images were acquired using Leica Hcx PL 100 × 1.30 or 63 × 1.32 oil objectives, and data were analysed using Fiji software. Vesicle tracking was performed on the indicated channels across 100 or 50 consecutively collected frames using TrackMate for Fiji (vesicle diameter = 1 μm; thresholds and other parameters were chosen as appropriate based on control samples; the same parameters were used across all samples and experiments within a given group).

Fractional distances were calculated as follows. Fluorescence intensities along multiple line ROIs (assessed by using the line profile tool of LAS-AF) were normalized to median, and background pixels were excluded from the analysis by determining the signal threshold. Distances corresponding to the remaining (vesicular) pixels were plotted as fractions of the distance from the nucleus to the plasma membrane along the same line.

**Fluorescence recovery after photobleaching**

Fluorescence recovery after photobleaching (FRAP) experiments on Rab7 were performed using MelJuSo cells stably expressing ectopic GFP-Rab7, and those on TBC1D15 were performed on endogenous GFP-TBC1D15 in HeLa cells. Leica’s LAS-X FRAP wizard software in combination with a Leica SP8 WLL or SP5 microscope equipped with a climate chamber was used for image acquisition. A pre-bleach image was collected using 10% laser power. Subsequently, photobleaching was performed on a single endosome or mitochondria-containing spots for 0.75 s at 100% laser power, resulting in a 90–100% reduction of the fluorescence signal. Sequential images were then collected at 10% laser power at 5-s or 0.4-s intervals for 20–60 frames. Image analysis was performed using ImageJ. Maximum fluorescence recovery (i.e. fluorescence prior to bleaching) was set at 100%, while post-bleach values were corrected for background and bleaching effects (Reits & Neefjes, 2001).

**Correlative light and electron microscopy (CLEM) of lysosomal distribution and ultrastructure**

HeLa cells expressing endogenous CD63-GFP were grown to a density of 3 × 10^5 cells per coverslip. Cells were incubated with SiR-Lysosome (Spirochrome) in complete DMEM for 30 min before fixation in 1 × PHEM buffer containing 4% PFA and 0.1% glutaraldehyde. Fluorescence imaging of fixed cells was performed using a Deltavision RT widefield microscope system (GE Healthcare) equipped with a 100/1.4NA oil immersion lens and a Cascade II EM-CCD camera (Photometrics). Multi-channel Z-stacks were captured and deconvolved using Softworx 6.5.2 (GE Healthcare). After fluorescence imaging, cells were flat embedded for electron microscopy using established protocols as previously described (Oorschot et al., 2002). Cells were postfixed with 1% OsO4 with 1.5% K4Fe(II)(CN)6 in 1 × PHEM for 1 h on ice, followed by washing steps in ddH2O and stained with 2% uranyl acetate in ddH2O at room temperature, followed by further washing steps with ddH2O.

Finally, samples were subjected to a graded ethanol series for dehydration. After dehydration, samples were embedded in Epon resin (ratio: 12 g Glycid Ether 100, 8 g dodecenylsuccinic anhydride, 5.5 g methylnadic anhydride, 560 μl N-benzylidymethyamine). After Epon polymerization, the resin blocks were removed from the coverslips and prepared for EM as reported before with slight modifications (Fermie et al., 2018). Regions of interest selected based on fluorescence imaging were cut out as blocks, from which serial sections (70 nm or 300 nm) were made with an ultramicrotome (Leica) and collected on carbon coated copper TEM grids. Thin sections were imaged using a Tecnai T12 TEM (Thermo Scientific), equipped with Veleta (EMSIS GmbH). Stitched EM images were collected by SerialEM: https://www.cambridge.org/core/journals/microscopy-and-microanalysis/article/serialem-a-program-for-automated-tilt-series-acquisition-on-tecnai-microscopes-using-prediction-of-specimen-position/DBSEA0250C3803C2D7C145CD13B4ADE6 and processed by IMOD software. Tomograms from 300 nm sections were made using a Tecnai 20 TEM (Thermo Fisher Scientific) operating at 200 kV, equipped with an Eagle 4K × 4K CCD camera running Xplore3D (Thermo Fisher Scientific) software. Single tilt image series were automatically collected with 1° tilt increments from −60° to +60°. Tomographic reconstructions were generated using IMOD software (Mastronarde & Held, 2017).

**Immunogold EM**

HeLa cells transfected as indicated were prepared for cryosectioning, as described (Peters et al., 2006; van Elsland & van Kasteren, 2016). Briefly, cells were fixed for 24 h in freshly prepared 2% paraformaldehyde in 0.1 M phosphate buffer. Fixed cells were scraped, embedded in 12% gelatin (type A, bloom 300, Sigma) and

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cut with a razor blade into 0.5-mm³ cubes. The sample blocks were infiltrated in phosphate buffer containing 2.3 M sucrose. Sucrose-infiltrated sample blocks were mounted on aluminium pins and plunged in liquid nitrogen. The vitrified samples were stored under liquid nitrogen. Ultrathin sections of 75 nm were obtained essentially as described elsewhere (van Elsland & van Kasteren, 2016). Briefly, the frozen sample was mounted in a cryo-ultramicrotome (Leica). The sample was trimmed to yield a squared block with a front face of about 400 × 300 μm (Diatome trimming tool). Using a diamond knife (Diatome) and antistatic devise (Leica), a ribbon of 75-nm-thick sections was produced that was retrieved from the cryo-chamber with the lift-up hinge method (Bos et al, 2011). A droplet of 2.3 M sucrose was used for section retrieval. Obtained sections were transferred to a specimen grid previously coated with formvar and carbon. Grids containing thawed cryo sections of cells fixed with 2% paraformaldehyde were incubated on the surface of 2% gelatin at 37°C. Subsequently, sections were rinsed to remove the gelatin and sucrose, blocked with 1% BSA in PBS and then double-labelled with the indicated antibodies and 10-nm and 15-nm protein A-coated gold particles (CMC, Utrecht University) (Slot et al, 1991). Next, grids were embedded in 1.8% methylcellulose and 0.6% uranyl acetate. EM imaging was performed with an Tecnai 20 transmission electron microscope (FEI) operated at 120 kV acceleration voltage.

**Protein expression and purification**

Recombinant protein expression of GST-Rab7, GST-Arl8b and GST-Rab5 was performed in the E. coli Rosetta2 (DE3) strain. Briefly, transformed cultures were inoculated in LB at 37°C till OD₆₀₀ 0.6 and expression was induced with IPTG at 16°C overnight. Cultures were harvested in 50 mM HEPES pH 7.5, 250 mM NaCl, 1 mM EDTA and 1 mM DTT, freshly supplemented with complete EDTA-free protease inhibitors (Roche Diagnostics) and lysed by microtip sonication. For purification of GST-fusion proteins, lysates were incubated with Glutathione Sepharose 4B affinity resin (GE Healthcare). Recombinant proteins were eluted with glutathione, and eluates were purified by size-exclusion chromatography using a Superdex™ 75 (16/600) gel filtration column (GE Healthcare) equilibrated with buffer 50 mM Tris–HCl pH 8.0, 150 mM NaCl. For purification of His-Rab9, lysates were incubated with prequillivaled Talon Co²⁺ resin (Clontech). The resin was then washed extensively with 20 mM HEPES pH 7.5, 200 mM NaCl and 8 mM β-mercaptoethanol, and His-Rab9 was eluted in washing buffer supplemented with 500 mM imidazole. His-TBC1D15 was purified from HEK293T cells lysed in lysis buffer (0.5% Triton X-100, 20 mM HEPES pH 7.5, 200 mM NaCl, 8 mM β-mercaptoethanol) freshly supplemented with complete EDTA-free protease inhibitors. Clarified supernatant was incubated with prequillivaled Talon Co²⁺ resin (Clontech), and the same washing and elution steps were followed as for His-Rab9 above.

Purity, apparent molecular weight and concentration of recombinant proteins were evaluated by SDS–PAGE followed by InstantBlue or Coomassie staining.

**In vitro γ³²P-GTP loading assays**

GTPase assays were performed as previously described (Askjaer et al., 1999). Briefly, GST-Rab7, GST-Rab5, His-Rab9 and GST-Ran were produced and purified as described in “Protein Expression and Purification”.

GTPases were loaded with [γ-³²P]GTP (10 mCi/ml, > 5,000 Ci/mmol; GE Healthcare) in the presence of 10 mM EDTA. Subsequently, MgCl₂ was added to a final concentration of 20 mM, followed by gel filtration on a Bio-Spin 6 column (Bio-Rad) equilibrated with buffer (0.1 M Tris–HCl, pH 7.5, 10 mM MgCl₂, 2 mM DTT, 0.5 mM NaCl) to remove free [γ-³²P]GTP. Reaction mixtures containing GST-fusion proteins in reaction buffer (40 mM Tris–HCl, pH 8.0, 50 mM NaCl, 8 mM MgCl₂, 1 mM DTT, 0.5 mM non-radioactive GTP, 0.1 mg/ml BSA, 1 mM Na₃PO₄, 1% (v/v) glycerol) were assembled on ice and incubated with increasing concentrations of His-TBC1D15 for 15 min. Reactions were stopped by adding 1 ml of charcoal suspension (7% w/v charcoal, 10% v/v ethanol, 0.1 M HCl, 10 mM KH₂PO₄), and the mixture was centrifuged for 5 min. Release of [³²P]-orthophosphate was quantified by liquid scintillation counting of the supernatant.

**Co-immunoprecipitation**

HEK293T cells were lysed for 30 min in lysis buffer (0.8% NP-40, 50 mM NaCl, 50 mM Tris–HCl pH 8.0, 5 mM MgCl₂) freshly supplemented with complete EDTA-free protease inhibitors (Roche Diagnostics). Supernatants obtained following centrifugation (10 min at 12,000 × g) were incubated with GFP-Trap_A, RFP-Trap_A or Myc-Trap_A agarose (Chromotek) rotating at 4°C for 1 h. Beads were then washed 4× in lysis buffer, ensuring that buffer was completely removed after the last wash using a needle before the addition of SDS Sample Buffer (containing 5% β-mercaptoethanol), followed by 5-min incubation at 95°C. Co-immunoprecipitated proteins were separated by SDS–PAGE for Western blotting as indicated.

**Proximity-based labelling (BioID)**

Proximity-based labelling experiments were performed as described before (Sapmaz et al., 2019). Briefly, HEK293T or HeLa cells were transfected with either 2xHA-TurboID vector control or 2xHA-TurboID SKIP and GFP-TBC1D15 (48 h following siRNA transfection, if applicable). After 24 h, the cells were treated with biotin (Sigma, B4639) at 50 μM final concentration for 5–6 h. Cells were lysed for 20 min in lysis buffer (0.8% NP-40, 150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 5 mM MgCl₂) freshly supplemented with protease inhibitors (Roche). SDS (1%) was then added to the supernatant following centrifugation (20 min, 4°C, 20,817 × g). High-capacity neutravidin beads (Thermo Scientific, 29202) were then added and samples were incubated rotating at 4°C overnight. Samples were washed 4× in buffer (0.8% NP-40, 150 mM NaCl, 50 mM Tris–HCl pH 8.0, 1% SDS and 5 mM MgCl₂) and eluted with SDS Sample Buffer (containing 5% β-mercaptoethanol), followed by 5-min incubation at 95°C. Biotinylated proteins were separated by SDS–PAGE and detected by Western blotting.

**SDS–PAGE and immunoblotting**

Samples were separated by 8% or 10% acrylamide gel and stained using InstantBlue (Expedeon) or transferred to Nitrocellulose (0.45 μm, GE Healthcare) or Immobilon-P PVDF membrane (0.45 μm, Millipore) at 300 mA for 3 h. Membranes were blocked...
in 5% milk (skim milk powder, LP0031, Oxioid) in 1× PBS (P1379, Sigma-Aldrich), incubated with primary antibodies diluted in 5% milk/0.1% PBS-Tween-20 (PBST) for 1 h, washed 3 × 10 min in 0.1% PBST, incubated with the secondary antibody diluted in 5% milk/0.1% PBST for 30 min and washed 3× in 0.1% PBST. Antibody signals on Nitrocellulose membranes were detected using Odyssey CLx (LI-COR), and those on PVDF membranes were collected on an Amersham Imager 600 (GE Healthcare).

Statistics

All error bars correspond to SD of the mean. Statistical evaluations report on Student’s test (analysis of two groups) or one-way ANOVA analyses (analysis of three or more groups), as described in the corresponding figure legends. *P < 0.05, **P < 0.01, ***P < 0.001, ns: not significant and nd: not determined.

Expanded View for this article is available online.

Acknowledgements

We thank S. Munro (MRC) for the generous gift of Aril8b-GFP and SKIP cDNA, as well as M. Seaman (CIMR) and T. Johansen (IMB) for kindly providing GFP-TBC1D5 and GFP-FYCO1 constructs, respectively. We thank R. Kim of the LUMC protein facility for help with secondary structure prediction in designing the SKIP truncation mutants, B. Koster for access of experiments. BC performed in vitro interaction assays, NL and JF carried out CLEM studies, DE performed immunoEM, RHW identified the KMI motif in SKIP, and CK performed GTPase assays. JLLA, SYZ, LJ and DH provided technical support. JK and RK advised on the project. IB, JB and MLM wrote the manuscript with input from JN. JN and IB jointly coordinated the study.

Author contributions

MLMJ, JB and IB designed the study and performed and analysed the majority of experiments. BC performed in vitro interaction assays, NL and JF carried out CLEM studies, DE performed immunoEM, RHW identified the KMI motif in SKIP, and CK performed GTPase assays. JLLA, SYZ, LJ and DH provided technical support. JK and RK advised on the project. IB, JB and MLM wrote the manuscript. JN and IB jointly coordinated the study.

Conflict of interest

The authors declare that they have no conflict of interest.

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