Late endosomal transport and tethering are coupled processes controlled by RILP and the cholesterol sensor ORP1L

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Summary
Late endosomes and lysosomes are dynamic organelles that constantly move and fuse to acquire cargo from early endosomes, phagosomes and autophagosomes. Defects in lysosomal dynamics cause severe neurodegenerative and developmental diseases, such as Niemann–Pick type C disease and ARC syndrome, yet little is known about the regulation of late endosomal fusion in a mammalian system. Mammalian endosomes destined for fusion need to be transported over very long distances before they tether to initiate contact. Here, we describe that lysosomal tethering and transport are combined processes co-regulated by one multi-protein complex: RAB7–RILP–ORP1L. We show that RILP directly and concomitantly binds the tethering HOPS complex and the p150 Glued subunit of the dynein motor. ORP1L then functions as a cholesterol-sensing switch controlling RILP–HOPS–p150 Glued interactions. We show that RILP and ORP1L control Ebola virus infection, a process dependent on late endosomal fusion. By combining recruitment and regulation of both the dynein motor and HOPS complex into a single multiprotein complex, the RAB7–RILP–ORP1L complex efficiently couples and regulates the timing of microtubule minus-end transport and fusion, two major events in endosomal biology.

Key words: Endosomal fusion, Ebola virus, HOPS complex, RAB7, RILP, ORP1L, Dynein

Introduction
Intracellular organelles continuously move and fuse due to the activities of motor proteins and fusion complexes. Fusion of organelles occurs in a series of sequential steps starting with transport of organelles along the cytoskeleton towards each other. Subsequently, these organelles tether, dock and fuse to exchange cargo (Bröcker et al., 2010). Early endosomes, autophagosomes and phagosomes fuse with acidic late endosomes and lysosomes (LE) in order to degrade endosomal cargo and recycle nutrients. Defects in the lysosomal transport and fusion machinery result in severe neurodegenerative and developmental diseases such as Niemann–Pick type C disease (Sobo et al., 2007; Rocha et al., 2009), Charcot–Marie–Tooth disease (CMT) (BasuRay et al., 2010; Spinosi et al., 2008), classical juvenile onset neuronal ceroid lipofuscinosis (Usui-Rauva et al., 2012) and arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome (Gissen et al., 2004; Cullinane et al., 2010).

Much of our understanding of LE fusion is derived from studies in yeast where vacuolar (lysosomal) tethering is orchestrated by the HOMotypic fusion and vacuole Protein Sorting (HOPS) complex that subsequently interacts with SNARE proteins to induce fusion (Ostrowicz et al., 2010). In contrast to yeast cells, LE in mammalian cells first need to be transported over long distances before they can meet, tether and fuse to exchange cargo. LE transport is well studied in mammalian cells, but tethering for fusion as well as the cross-talk between transport and tethering processes are poorly understood.

In the endo-lysosomal system, various small GTPases of the Rab and Arf family facilitate transport by recruitment of relevant motor proteins from the dynein, kinesin or myosin families (Hoefnagel et al., 2005; Wu et al., 2002; Paul et al., 2011; Kuilj et al., 2007; Stenmark, 2009) that specify directionality. The Rab7 effector RILP interacts with the p150 Glued subunit of the dynein–dynactin motor complex thus controlling microtubule minus-end transport and fusion. ORP1L then functions as a cholesterol-sensing switch controlling RILP–HOPS–p150 Glued interactions. We show that ORP1L controls Ebola virus infection, a process dependent on late endosomal fusion. By combining recruitment and regulation of both the dynein motor and HOPS complex into a single multiprotein complex, the RAB7–RILP–ORP1L complex efficiently couples and regulates the timing of microtubule minus-end transport and fusion, two major events in endosomal biology.
While the spatial control of motor protein recruitment to vesicles is understood at some level of detail, this is not the case for tethering and subsequent fusion of vesicles. Tethering factors function to physically bridge two opposing membranes, establish quality control (i.e. prevent fusion between unrelated compartments) before recruiting SNARE proteins for actual membrane fusion. RAB GTPases are also involved in vesicle tethering and fusion (Bröcker et al., 2010). The RAB5 effector EEA1 can promote docking and fusion of early endosomes (Christoforidis et al., 1999) and other RAB GTPases support multisubunit tethering complexes at the ER, Golgi and the plasma membrane (Bröcker et al., 2010). Late endosomal tethering is best understood in yeast where vacuolar (lysosomal) tethering is orchestrated by the HOPS complex comprising six vacuolar protein-sorting (Vps) components: Vps18, Vps11, Vps16, Vps41, Vps39 and Vps33 (Wickner, 2010). Homologues of all these Vps subunits are found in mammalian systems with some expansion. Specifically two VPS33 proteins exist – VPS33A and VPS33B – and a new VPS33B-interacting protein has evolved; SPE-39 (Huizing et al., 2001; Zhu et al., 2009; Gissen et al., 2005). The yeast RAB7 homologue Ypt7 recruits and assembles the HOPS complex by interacting with Vps39 and Vps41 on different sides of the HOPS complex, suggesting that HOPS bridges Ypt7 on two vesicles (Cabrera et al., 2009; Bröcker et al., 2012).

In yeast, the HOPS complex is the only known Ypt7 effector and no RILP homologue is found. In contrast to mammals, the dynein motor appears to not be essential for late endosomal tethering in yeast, indicating that interactions around Ypt7/RAB7 may be different between yeast and human with respect to vesicular transport and tethering functions. Despite significant evolutionary conservation of the actual tethering complex from yeast to human, the current understanding of the HOPS complex composition, recruitment and function in mammalian systems remains limited. Human HOPS has been implicated in EGFR routing (Chirivino et al., 2011) and autophagosome maturation (Liang et al., 2008), and is critical for efficient cytosolic entry of Ebola virus from NPC-1 containing late endosomes (Carette et al., 2011). Mutations in the additional HOPS subunits VPS33b and SPE-39 cause complex multiorgan failures, such as ARC syndrome, which is characterized by impairments in granule secretion and membrane fusion (Gissen et al., 2004; Cullinan et al., 2010). Another HOPS interactor, the small GTPase ARL8b controls lipid loading of CD1 molecules in lysosomes through interactions with some subunits of the HOPS complex (Garg et al., 2011). How the mammalian HOPS complex is recruited to late endosomal membranes, is unclear.

Here, we demonstrate that RAB7 effector RILP recruits the mammalian HOPS complex to late endosomal membranes, and an ARC syndrome disease mutant of VPS33b fails to be recruited to RILP. RILP simultaneously binds both the HOPS complex and dynein motor, thereby coupling transport and tethering for fusion in one protein complex. Uncoupling these processes has major effects on cargo delivery as illustrated for Ebola infectivity. The cholesterol sensor ORP1L controls RILP–HOPS–dynein-motor interactions and times both transport and tethering. Collectively, our observations suggest that two main aspects of late endosomal life – transport and fusion – are integrated and regulated by a single RAB7–RILP–ORP1L protein complex to promote efficient transport and delivery of late endosomal cargo.

Results

RILP induces late endosomal tethering in the absence of functional dynein motor

The RAB7 effector RILP recruits the p150Glued subunit of the dynein–dynactin motor complex to late endosomal membranes to induce microtubule minus-end transport (Cantalupo et al., 2001; Jordens et al., 2001). Expression of RILP in cells induces late endosomal clustering at the microtubule-organizing centre (MTOC) of cells (Rocha et al., 2009; Cantalupo et al., 2001; Jordens et al., 2001) (Fig. 1A) due to dynein motor activity. As active GTP-bound Ypt7 (the yeast RAB7 homologue) is involved in late endosomal tethering (Cabrera et al., 2009) and RILP locks RAB7 in an active GTP-bound state, we wondered whether RILP present on LE might also induce its tethering. To test this, we expressed RILP in cells where dynein motor activity was abrogated by overexpressing p50dynamin (Fig. 1A) or silencing the dynein chain (DHC) (Fig. 1B). As DHC is 500 kDa and difficult to assess by SDS-PAGE, protein expression was determined by its surrogate marker dynein intermediate chain (DIC) that is stable only in presence of DHC. DIC was efficiently eliminated when DHC was silenced. Under these conditions RILP-labelled endosomes remained in one or two large clusters per cell. These data show that RILP induces LE clustering even in the absence of functional dynein motor, and that RILP might be involved in tethering LEs. To visualize the clusters induced by RILP expression in higher resolution, MelJuSo cells were transfected with GFP–RILP, fixed and sections stained with anti-GFP (10-nm gold) antibodies for analysis by electron microscopy. RILP was often observed at contact sites between LE (Fig. 1C), again suggesting a link between RILP and tethering. LE tethering in yeast is achieved by the HOPS complex which is recruited to LE membranes via the small RAB GTPase Ypt7. To test the localization of the HOPS complex in mammalian cells, we reconstituted a VPS11 knockout (KO) cell line (Carette et al., 2011) with GFP–VPS11. GFP-tagged VPS11 could be successfully incorporated into high molecular mass complexes similar to their endogenous equivalents as assessed by gel filtration and western blotting (Fig. 1D) and a cytosolic distribution was observed for VPS11 (Fig. 1E). To test whether RILP promotes HOPS recruitment to late endosomes, we co-expressed RILP in the GFP–VPS11-expressing cells. In this case, we observed a striking recruitment of VPS11 to RILP-labelled late endosomes (Fig. 1E). While the C-terminus of RILP binds and locks RAB7 in a GTP-bound state, the N-terminus of RILP directly binds the dynein motor (Johansson et al., 2007). Expression of an N-terminally truncated RILP mutant (RILPΔ199) in GFP–VPS11 expressing cells failed to recruit VPS11 to LE (Fig. 1E). These data suggest that the N-terminus of RILP, recruits the dynein motor and is also involved in recruitment of the HOPS complex to late endosomes for tethering.

Mammalian HOPS is recruited to membranes by RILP and regulated by ORP1L

To study localization of the other mammalian HOPS complex subunits, we co-expressed the eight (tagged) HOPS complex subunits in the melanoma cell line MelJuSo (S1A, S1B). Expression levels were moderate (supplementary material Fig. S1C) and the epitope-tagged proteins were successfully incorporated into high molecular mass complexes similar to their endogenous equivalents as determined by gel filtration.
A cytosolic distribution for all of the HOPS complex subunits was observed upon ectopic expression. Additional expression of constitutively active RAB7 did not suffice to induce recruitment of HOPS to LE (Fig. 2A; supplementary material Fig. S1A,B). However, when co-expressed with RILP (supplementary material Fig. S1A,B) seven of the eight HOPS subunits were recruited to RILP-decorated late endosomes. Only VPS33a was not recruited to RILP unless its interaction partner VPS16 was co-expressed (supplementary material Fig. S1B). Mutations in one of the HOPS complex subunits, VPS33b, causes ARC syndrome and one disease mutant of VPS33b (L30P), has been reported to be deficient in membrane recruitment (Gissen et al., 2004). Indeed, the latter VPS33b mutant failed to be recruited by RILP to late endosomes (Fig. 2B). To further confirm interactions between HOPS and RILP, we performed co-immunoprecipitation experiments. RILP could be co-isolated with VPS11, VPS18 as well as SPE-39 (Fig. 2C).

Binding of the dynein motor to RAB7–RILP is controlled by the cholesterol sensor ORP1L (Rocha et al., 2009). ORP1L also binds RAB7 and prevents binding of the dynein motor to RILP under low cholesterol conditions, as ORP1L then binds to ER protein VAP-A which removes p150Glued from RILP (Rocha et al., 2009). To test if ORP1L also controls RILP-HOPS interactions, we co-expressed various subunits of the HOPS complex with RILP and wild-type ORP1L or a mutant of ORP1L.
mimicking low cholesterol conditions (ORP1L\textsuperscript{\textordmasculine K/D}) that releases p150\textsuperscript{Glued} from the RAB7–RILP complex (Fig. 2D; supplementary material Fig. S1E,F). wtORP1L allowed binding of all subunits of the HOPS complex to RILP-labelled endosomes unlike ORP1L\textsuperscript{\textordmasculine K/D} that effectively prevented HOPS subunit recruitment to endosomes (Rocha et al., 2009). Under low cholesterol conditions, ORP1L changes conformation to expose an FFAT motif that subsequently interacts with VAP-A to remove the dynein motor from RAB7–RILP (Rocha et al., 2009). To test whether VAP-A is involved in removing the HOPS complex from RILP under low cholesterol conditions, we repeated the experiments with an ORP1L mutant: ORP1L\textsuperscript{\textordmasculine K/D}FFAT(D478A) that is similar to ORP1L\textsuperscript{\textordmasculine K/D} with a single mutation in the FFAT domain to abolish interactions with ER protein VAP-A (Rocha et al., 2009) (supplementary material Fig. S1G). This ORP1L mutant again allowed recruitment of HOPS complex subunits to RILP labelled endosomes suggesting that HOPS binding to RILP is – like p150\textsuperscript{Glued} binding – controlled by cholesterol sensor ORP1L through ER protein VAP-A. Together our data indicate that the HOPS complex is recruited to membranes...
Fig. 3. See next page for legend.
by the RAB7 effector RILP and that recruitment is further regulated by the cholesterol sensor ORP1L.

**Fine-mapping of HOPS subunit recruitment to the N-terminus of RILP**

In the yeast HOPS complex there are multiple binding sites for Ypt7 (the yeast RAB7 homologue) (Bröcker et al., 2012). To define whether the mammalian HOPS complex interacts with multiple sites on RILP, N-terminal RILP truncations were constructed and expressed with different HOPS complex subunits in MelJuSo cells (Fig. 3A; supplementary material Fig. S2B; quantified in Fig. 3B). Expression levels of the various RILP constructs were similar and all RILP mutants localized properly to late endosomes (supplementary material Fig. S2A). All HOPS subunits [except for VPS33a (supplementary material Fig. S2C)] were recruited to the N-terminal 199 amino acids of RILP that also binds the dynein-motor-complex subunit p150Glued (Johansson et al., 2007). We observed that different HOPS complex subunits were recruited to different regions of RILP (summarized in Fig. 3D). As HOPS subunits were overexpressed, these should be interactions of free HOPS subunits to the different RILP deletion mutants. Recruitment of the entire HOPS complex to RILP probably requires multiple binding sites as deletion of RILP aa 1–100 (disrupting recruitment of VPS11 and VPS16) also negatively affected recruitment of VPS18 (Fig. 3B). These experiments were further confirmed in biochemical experiments where VPS18 was co-isolated with different RILP truncation mutants (Fig. 3C; supplementary material Fig. S2D). While RILPA199 failed to co-isolate VPS18, RILPA150 and smaller deletion mutants were successful, as also shown in Fig. 3B. Together these data indicate that the mammalian HOPS complex is recruited to late endosomes by multiple interactions with the N-terminal half of the RAB7 effector RILP, the same region that accepts the p150Glued subunit of the dynein motor.

**RILP concomitantly binds the dynein motor and HOPS complex**

The p150Glued subunit of the dynein motor binds directly to RILP (Johansson et al.). To test if the interactions between RILP and HOPS subunits are also direct, we assayed *in vitro* the binding of two HOPS subunits (VPS18 and VPS41) to RAB7 and/or RILP by SPR. The recombinant proteins used (Fig. 4A) were incorporated into higher molecular mass complexes running at similar complex sizes to endogenous HOPS, indicating that the proteins are well-folded and can interact with their endogenous partners (Fig. 4B). The N-terminus of VPS18 (as for the soluble isolated protein) was shown to be sufficient for recruitment to late endosomes by RILP (supplementary material Fig. S3A) and RAB7/RILP assembled into a stable complex for the SPR experiments (supplementary material Fig. S3B). The recombinant soluble HOPS subunits GST-VPS18 (aa 1–580) and GST–VPS41 were bound to a surface plasmon resonance (SPR) chip and responses were determined in the presence of GTP at different concentrations of RAB7, RILP, the RAB7–RILP complex or the C-terminus of p150Glued (C25) (Fig. 4C). These values were corrected for a GST-only control and from the SPR response curves the equilibrium association constant $K_a$ (1/$K_d$) was determined (Fig. 4C). GTP-loaded RAB7 associated to VPS18 and VPS41 with a low but detectable affinity (4.12 x 10$^4$ and 5.80 x 10$^4$ M$^{-1}$, respectively). Binding affinities of RILP to the same HOPS subunits was ~10-fold higher (3.41 x 10$^5$ and 5.39 x 10$^5$ M$^{-1}$, respectively). The RAB7–RILP complex had a further 2–3 fold increase in affinity for VPS18 and VPS41 (5.98 x 10$^5$ and 1.13 x 10$^6$ M$^{-1}$, respectively). No productive binding was detected for VPS18 and VPS41 to C25 ($K_a$ < 1 x 10$^4$ M$^{-1}$). Together our data indicate that both HOPS complex subunits and the C-terminus of p150Glued (Johansson et al., 2007) can associate to RILP in cells and *in vitro*. This suggests that two general processes in late endosomal life – fusion and transport – are directly coupled through the RAB7-effector RILP. To test this, GFP-tagged VPS41 was co-expressed with RILP and cells were subsequently stained for the dynein motor subunit p150Glued. RILP recruited both p150Glued and VPS41 to clustered endosomes (Fig. 4D). Similar results were obtained with VPS18 (Fig. 4D). To further address whether HOPS and p150Glued bind to RILP simultaneously or mutually exclusive (compete for the same site on RILP), RAB7–RILP and RAB7–RILP–C25 complexes were reconstituted using purified recombinant RAB7, RILP and the C-terminus of p150Glued (C25). Their binding to VPS18 (aa1–580) and VPS41 subunits of the HOPS complex was assessed by SPR (Fig. 4E). Whereas p150Glued–C25 failed to interact with the HOPS subunits, RAB7–RILP–C25 associated with a higher binding affinity than RAB7–RILP only [$K_a$ VPS18 (aa1–580); 1.22 x 10$^6$ M$^{-1}$, VPS41; 1.67 x 10$^6$ M$^{-1}$]. This suggests that the C-terminal part of p150Glued supports, rather than competes for, the interaction of the RAB7–RILP complex to HOPS subunits and that p150Glued and HOPS subunits can simultaneously bind to RILP, effectively integrating a motor protein for transport and a tethering complex for fusion on a single late endosomal receptor: RAB7–RILP.

**Late endosomal tethering by RILP and HOPS is required for Ebola virus infectivity**

RILP couples late endosomal minus-end transport by the dynein motor complex and tethering by the HOPS complex. Overexpression of RILP induces late endosomal clustering at the MTOC and this clustering is a consequence of p150Glued–dynein-motor recruitment (driving minus-end transport) (Cantalupo et al., 2001; Johansson et al., 2007; Jordens et al., 2001) and HOPS complex recruitment (for vesicle tethering)
To study the relative contribution of RILP–HOPS interactions in late endosomal dynamics, we defined RILP mutants that allowed interactions with p150Glued but not with the HOPS complex. To define critical position in RILP to uncouple both processes, we first determined charged amino acids (these are likely exposed to the protein surface) in the N-terminus of RILP and conserved between a series of species. We reversed the charge (for example, R to E or E to R) and tested for recruitment of p150Glued or HOPS subunits (supplementary material Fig. S4A–H). Most mutations that affected RILP interactions with...
cells expressing mRFP–RILP(D125RE126R) relative to control cells expressing either mRFP only or mRFP-tagged wild-type RILP when infection was assessed by microscopy (Fig. 5C,D) or flow cytometry (supplementary material Fig. S4J). When late endosomal recruitment of both p150\textsubscript{Glued} and HOPS was prevented with RILPA199, inhibition of infection of rVSV-GP-EboV remained attenuated to a level similar as inhibition of HOPS recruitment alone.

As the cholesterol sensor ORP1L acts as regulator of HOPS as well as dynein motor binding to RILP (Rocha et al., 2009) (Fig. 2D), we tested whether ORP1L also affected Ebola virus infection. Cells were transfected with mRFP, mRFP–ORP1L or mRFP–ORP1LAORD (this mutant abolish RILP–HOPS and RILP–p150\textsubscript{Glued} interactions) before infection with rVSV-GP-EboV (Fig. 5E). A 50% reduction in rVSV-GP-EboV infectivity in cells expressing mRFP–ORP1LAORD relative to control cells expressing either mRFP only or mRFP-tagged wild-type ORP1L was observed (Fig. 5E). Collectively, our data suggest that RAB7–RILP is the late endosomal receptor for the HOPS complex and p150\textsubscript{Glued} and that binding of these complexes to RILP is timed by the cholesterol sensor ORP1L to regulate endosomal maturation.

**Discussion**

The life of a vesicle can be described in a few general terms, including membrane transport and fusion. The small GTP-binding proteins of the Rab/Ypt family have been defined as key regulators of these processes (Stenmark, 2009). One member, RAB7 acts as the late endosome receptor for the dynein motor via its effector RILP (Jordens et al., 2001). In yeast, the RAB7 homologue Ypt7 recruits the HOPS complex to membranes to control correct interaction of late endosomes with vacuoles prior to fusion (Bröcker et al., 2010). Homologues for all yeast HOPS subunits have been found in eukaryotic genomes, and two additional homologues VPS33b and SPE-39 (VPS16b/VIPAR) exist. Deletion or mutations of HOPS subunits deep orange (VPS18 homologue), carnation (VPS33a homologue) or dpsy16a (VPS16 homologue) result in defective late endosomal and lysosomal biogenesis in *Drosophila melanogaster* (Pulipparacharuvil et al., 2005; Sevrioukov et al., 1999). Mutations in VPS41 can protect against α-synuclein-induced neurotoxicity in *C. elegans* and Parkinson’s disease in mammalian model systems (Harrington et al., 2012; Ruan et al., 2010), and VPS41-deficient mouse embryos fail to develop beyond the gastrulation stage (Aoyama et al., 2012). In addition, the HOPS complex has been implicated in Ebola virus infection, EGFR trafficking, autophagolysosome formation and endosomal peptide loading of CD1d (Carette et al., 2011; Ganley et al., 2011; Chirivino et al., 2011; Garg et al., 2011). Mutations in the additional HOPS subunits VPS33b and SPE-39 cause complex multiorgan failures such as ARC syndrome, which is characterized by impairments in granule secretion and membrane fusion (Gissen et al., 2004; Cullinane et al., 2010).

Although the HOPS complex is involved in all these processes, how the HOPS complex is regulated and recruited to late endosomal membranes in mammalian cells remains unclear.

Here we report that in mammals HOPS subunits are not recruited by RAB7 but by the RAB7 effector RILP. Using surface plasmon resonance (SPR) experiments, we demonstrate that HOPS subunits (VPS18 and VPS41) exhibit low affinity for RAB7 alone, 10–15-fold higher affinity for RILP and even higher affinity for RILP associated to RAB7. In evolution, HOPS

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**Fig. 4.** Simultaneous binding of HOPS subunits and p150\textsubscript{Glued} to RILP.

(A) Coomassie-stained SDS-PAGE gels with the indicated recombinant proteins used for the SPR experiments. The position of marker proteins is shown. (B) The recombinant proteins used in SPR studies are incorporated into higher molecular mass complexes. Recombinant GST–VPS41, GST–VPS18(1–512) or His–RILP were incubated with lysates of MelJuSo cells and lysates were subsequently size separated using gel filtration. Fractions were subjected to SDS-PAGE and western blotting with the indicated antibodies to detect complexes containing the recombinant proteins. (C) GST–VPS18 (aa 1–580) or GST–VPS41 was immobilized to an SPR chip, and binding of RAB7Q76L, RILP, a RAB7Q67L–RILP complex and C25 in the presence of GTP–cS was determined. RAB7Q76L and RILP were pre-incubated with GTP–cS to assemble the complex. The left panels show the responses (corrected to that of GST only) at different concentrations of RAB7, RILP or the RAB7–RILP complex. Right panel: K\textsubscript{D} (mean+s.e.m.) calculated from the SPR responses. Shown is K\textsubscript{D} of RAB7Q76L, RILP or RAB7Q67L–RILP binding *in vitro* to VPS18 (aa 1–580) or VPS41, all measured in the presence of GTP–cS. (D) MelJuSo cells expressing HA–RILP and GFP–VPS18 or GFP–VPS41 (as indicated) were fixed, immunolabelled with anti-p150\textsubscript{Glued} and anti-HA antibodies and imaged by CLSM. The zoom images are the boxed area from the merge images and illustrate co-localization of RILP with p150\textsubscript{Glued} and VPS18 or VPS41. Scale bars: 10 μm. Representative images from n>100 are shown. (E) Left panels: *in vitro* binding of GST–VPS18 (aa 1–580) or GST–VPS41 immobilized on a SPR chip to RAB7Q76L, RILP, the RAB7Q76L–RILP complex or the RAB7–RILP–C25 (the C-terminal domain of p150\textsubscript{Glued} complex (mean+s.e.m.). Binding was determined in the presence of GTP–γS and corrected for binding to GST only coupled to the SPR chip. Right panel: K\textsubscript{D} calculated from the SPR responses shown in B. Shown is *in vitro* binding of the C-terminal p150\textsubscript{Glued} fragment C25, the preformed complex of RAB7Q76L–RILP and RAB7Q67L–RILP–C25 to VPS18 (aa 1–580) and VPS41, all measured in the presence of GTP–γS (mean+s.e.m.).
recruitment has apparently shifted from RAB7/YPT7 to its effector RILP. RILP also binds the p150 Glued subunit of the dynein motor for minus-end-directed microtubule transport (Jordens et al., 2001; Cantalupo et al., 2001). We have mapped recruitment of HOPS to the N-terminus RILP and revealed that there are multiple positions in RILP required for HOPS recruitment.

**Fig. 5. RILP induces late endosomal tethering.** (A) Quantification of HOPS subunit recruitment to wild-type RILP (WT, black) and RILP D125RE125R (gray). Mean correlation coefficients (+s.e.m.) are shown for indicated HOPS subunits. *P<0.05, n>20 for each condition. (B) MelJuSo cells expressing HA–RILP, HA–RILPE93RE94R, HARILPD125RE126R and either co-expressing mRFP-p50 dynamitin or not were fixed, immunolabelled with anti-HA antibodies (green) and DAPI (blue) and imaged by CLSM. Representative images from n>100 are shown. The quantification on the right shows the average number of individual clusters and/or vesicles per cell (means + s.d.). Cells with >10 clusters were set at 10. *P<0.05, P<0.1. (C) HAP-1 cells expressing mRFP, mRFP–RILP, mRFP–RILPD199 or mRFP–RILPD125RE126R were infected with rVSV-GP-EboV for 8 hours. The virus produces GFP in the host cytosol after successful infection, which is detected and quantified. Right panel: cells were identified, masked and overlayed using Cell Profiler. Red, transfected cells; green, cells with successful infection of rVSV-GP-EboV; yellow, transfected and infected cells expressing the mRFP-labelled constructs. (D) Quantification of mRFP, mRFP-RILP-, mRFP-RILPD199- or mRFP-RILPD125RE126R-expressing cells successfully infected by rVSV-GP-EboV as measured by cytosolic GFP expression (means+s.e.m.). *P<0.05. (E) HAP-1 cells expressing mRFP, mRFP-ORP1L or mRFP-ORP1L ΔORD were infected with rVSV-GP-EboV for 8 hours. After successful infection, the virus produces GFP in the cytosol which is detected and quantified by flow cytometry. Shown is the percentage of transfected (mRFP-positive) and infected (GFP-positive) cells relative to transfected cells (mean+s.e.m). *P<0.05. Scale bars: 10 μm.
HOPS is an integral part of the RAB7–RILP–p150 Glued complex. This suggests that ER protein VAP-A. This terminates binding of the dynein motor and the HOPS complex as well as p150Glued to RILP. This mechanism shows how late endosomal cholesterol controls transport and fusion mediated by the complex of ORP1L, RAB7, RILP, dynein motor and HOPS.

As RILP coordinates vesicular tethering, it might modulate infectivity of pathogens entering the host via the endo-lysosomal pathway. For instance, Salmonella replicates in endosomes but avoids fusion with the degradative (phago)lysosome. Overexpression of RILP breaks this block and severely compromises intracellular bacterial infection (Guignot et al., 2004; Harrison et al., 2004; Marsman et al., 2004). Conversely, efficient delivery into late endosomes and lysosomes is essential for successful infection of Ebola virus by fusion and subsequent entry into the host cytosol. Here we show that RILP and ORP1L regulate fusion and infection by the Ebola virus. Various viruses including Dengue, influenza and HIV also have been reported to require late endosomal compartments for proper infectivity and propagation (van der Schaar et al., 2008; Caillet et al., 2011; Sieczkarski and Whittaker, 2003) and HIV propagation involves RILP as well (Lehmann et al., 2009; Lévesque et al., 2006). Whether late endosomal transport and tethering by RILP–HOPS–dynein is indeed important for host cell infection with these viruses remains to be determined.

VPS33b and SPE-39 have previously been reported to interact with RAB11A (Cullinane et al., 2010), a RAB GTPase present on recycling endosomes. Recycling endosomes have been implicated in epithelial polarization, and defective endosomal recycling might cause the abnormalities observed in polarized kidney and liver cells of ARC syndrome patients (Cullinane et al., 2010). We show that VPS33b and SPE-39 can also be recruited by RILP and that a disease mutant of VPS33b (VPS33b L30P) that causes severe ARC phenotype fails to be recruited to RILP. Our data suggest that VPS33b and SPE-39 might also have a role later in the endosomal pathway of mammalian cells.

Many cellular transport steps rely on dynein motor activity for transport and on multi-subunit tethering complexes for initiation of fusion (Stenmark, 2009; Bröcker et al., 2010) but the connection between these was unclear. We hypothesize that during evolution, organelles in higher eukaryotes may have required additional factors to recruit and time long-distance transport machineries, in addition to the tethering machinery.
reflected by two additional RAB7 effectors not found in yeast; RILP and ORP1L. These additional effectors might have interfered with existing RAB7 – HOPS interactions and this, in addition with the loss of the membrane binding motif in VPS41, might have required new interactions for membrane recruitment of the HOPS complex. This problem may have been solved by involving the RAB7-effector RILP (or a shared surface of RAB7–RILP) in HOPS recruitment which, at the same time, also provides cells with an efficient way of coupling transport and tethering and allowing co-regulation by the cholesterol sensor ORP1L. Whether transport and fusion processes are intricately coupled for other intracellular structures as well, remains to be determined.

Here, we have shown that the RAB7 effector RILP recruits protein complexes involved in two different but consecutive processes: the dynein-dynactin motor for minus-end transport of late endosomes and the HOPS complex for tethering of fusion of late endosomes and lysosomes. The cholesterol sensor ORP1L controls the timing of these processes, providing an additional layer of regulation (Fig. 6). Coupling these processes efficiently prepares incoming maturing vesicles for subsequent tethering and fusion steps. This provides cells with an efficient way of cargo delivery, as exemplified here by Ebola glycoprotein containing viral entry into the host cytosol from late endosomal compartments.

Materials and Methods

Constructs and cloning

RAB7, RILP, ORP1L, p50Giang and p50dynamin constructs have been described previously (Rocha et al., 2009; Jordens et al., 2001). GFP-VPS16 and GFP-VPS18 were gifts from Chengyu Liang (Li et al., 2008) (Department of Molecular Microbiology and Immunology of the University of Southern California, Los Angeles). Generation of other constructs is described below.

V5-VPS16 was generated by PCR using primerset1 (supplementary material Table S1) and inserted into the HINDIII – EcoRI sites in pcDNA3.1. VPS18 was subcloned into the XhoI-EcoRI sites of a 2FLAG-C1 vector. VPS33a was a gift of V. Faundez. VPS39 was a gift of J. Bonifacino (National Institutes of Health, Bethesda, MD) and was subcloned into an mRFP-C1 vector. Tag-VPS33b was generated by PCR (IMAGE CLONE 3449387) using primers set2 and was inserted into the EcoRI1 sites of mGFP-C1, mCHERRY-C1 and 2MYC-C1 Image vectors. Tag-VPS41 was generated by PCR (IMAGE CLONE 4822048) using primerset3 and was inserted into the BamH1 sites of mGFP-C1, mCHERRY-C1 and 2HA-C1. VPS11 was generated by PCR (IMAGE CLONE 6144541) using primer set4 and was inserted into the EcoRI1 site of mGFP-C1 or mCherry-C1. Spe-3/Cl40f1133-Tag was generated by PCR (IMAGE CLONE 3920459) using primerset set5 (fwd) and was inserted into the EcoRI1 and BamH1 sites of mGFP-N1 or mCHERRY-N1. Spe-39-FA was generated by PCR using primer set6 and was inserted into the BamH1 and EcoRI sites of a pcDNA3.1 vector.

Point mutants of RILP were generated by PCR using these respective primer sets; RILP E178R primerset7, RILP R159H160E60F primerset8, RILP D125R E126R primerset9, RILP E93R E94R primerset 10, RILP E60R primerset11, RILP R60F primerset12, RILP E38R primerset13, RILP Y26A primerset14.

Reagents

Rabbit anti-GFP and rabbit anti-mRFP antibodies were generated at home using purified His-mRFP or His-GFP recombinant proteins, respectively. Cross-reactivity has been excluded by Western blot analyses with various m-RFP- or GFP-labelled fusion proteins. Other antibodies used were: mouse anti-CD63 (Vector Laboratories).

Protein purification

His-RAB7Q76H, His–RILP and MBP-C25 were produced as previously described (Johansson et al., 2007; Rocha et al., 2009). Cells were lysed in 0.5 mM Trition X-100, 20 mM Heps, pH 7.5, 200 mM NaCl, 8 mM β-mercaptoethanol and complete EDTA-free protease inhibitor cocktail (Roche). The lysate was cleared by centrifugation and the supernatant was incubated with pre-equilibrated Talon Co5+ resin (Clontech Laboratories, Inc.) or MBP beads (New England Biolabs) for 30 minutes. The resin was washed extensively with 20 mM Heps pH 7.5, 200 mM NaCl and 8 mM β-mercaptoethanol. Proteins were aliquoted and stored at −80°C until used for protein reconstitution experiments.

Surface plasmon resonance

Binding assays were performed using Biacore T200 (GE). A CM5 Chip was coated with goat α-GST antibodies, and subsequently GST-tagged VPS proteins were immobilized in separate flow cells whereas GST protein alone was immobilized in a reference flow cell. Increasing concentrations of the analyte proteins were sequentially injected over the chip surface. Analysis of the data was done using Biacore T200 Evaluation Software (GE). Association constants were calculated using the equilibrium SPR response levels for each analyte concentration. Fitting of affinity constants and final figures applied Graphpad Prism (GraphPad Software, La Jolla, California, USA, http://www.graphpad.com).

Gel filtration

Transfected MelJuSo cells were washed with ice-cold PBS and scraped into buffer (50 mM Tris, 150 mM NaCl, 5 mM MgCl2, pH 7.4) supplemented with complete EDTA-free protease inhibitor cocktail. Cells were lysed by using the EMBL 8.020 mm ‘cell cracker’ homogenizer (ball sizes 8.0, 15 times) followed by a centrifugation for clearing membranes and nuclei. In experiments with recombinant proteins, untransfected MelJuSo cells were used and indicated recombinant proteins were mixed with the lysate at 30 minutes at 4°C. All supernatants were loaded onto a Superose 6 10 300 column connected to an Äkta purifier system (GE Healthcare). The column was equilibrated with running buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl) and the flow-rate of the column was set at 0.2–0.3 ml/minute. Fractions (1 ml) were collected and analysed by SDS-PAGE and western blotting with the indicated antibodies. Protein (complex) molecular masses were calculated by comparison of the respective elution volumes with protein markers with molecular masses ranging from 75 to 2000 kDa.

Melting curves

His–RAB7 and His–RILP were co-immunoprecipitated in the presence of 100 μM GTP·γ·S and melting curves were determined using SYPRO® Orange dye in Myq real-time PCR detection system (Biorad).

Transfection

Expression constructs were transfected using Effectene reagents (Qiagen) according to manufacturer’s instructions. For silencing, cells were transfected with Dharmacon’s (Thermo Fisher Scientific) anti-sRNA’s ON-TARGETplus SMARTpool against VPS16, VPS33a, VPS33b, VPS41, Dynem Heavy Chain or control sRNA (Thermo Fisher Scientific).

Cell culture and microscopy

MelJuSo cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) supplemented with 8% FCS in a 5% CO2 humidified culture hood at 37°C. All specimens were analyzed by confocal laser-scanning microscopes (CLSM, TCS-SP1, TCS-SP2, or AOBS; Leica) equipped with HCX Plan-Apochromat 63× NA 1.32 and HCX Plan-Apochromat 1.4 oil corrected objective lenses (Leica). The acquisition software used was LCS (Leica). For electron microscopy a CM10 electron microscope (FEI, Eindhoven, the Netherlands) was used.

Protein immunoprecipitation

MelJuSo cells were washed with ice-cold PBS and scraped into cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl2, 1% NP40, 10% glycerol, pH 7.4) supplemented with complete EDTA-free Protease Inhibitor Cocktail. Cell lysates were obtained by incubation on ice for 10 minutes followed by centrifugation for clearing. The supernatants were incubated for 2 hours with respective antibodies followed by capture with protein-G-Septarose-4 FF resin and washed extensively with wash-buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl2, 10% glycerol, pH 7.4). For GFP-tagged pull-down, GFP-TRAP beads were used (Chromotek).

Microscopy sample preparation

Transfected cells were fixed 4 hours post-transfection with ice-cold methanol (50% v/v isopropanol in 50% v/v methanol in PBS) for 15 minutes at −20°C. Cells were washed twice with PBS and fixed with 4% formaldehyde (in PBS) for 15 minutes, then permeabilized with 0.1% Triton X-100. Cells were then incubated with primary antibodies followed by Alexa Fluor secondary antibodies (Molecular Probes).
VPS11 rescue experiment

Haploid Hap! cells knockout for VPS11 (Carette et al., 2011) were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) supplemented with 8% FCS. Cells were electroporated with GFP-VPS11 and mRFP-VPS11 in combination with GFP-RILP, or GFP-RILPA199 and, after 24 hours, fixed with 4% formaldehyde in PBS for 30 minutes and mounted in Vectashield mounting medium (Vector Laboratories). The slides were analyzed by confocal laser-scanning microscopes.

Immunoelectron microscopy

Cells were fixed in 2% paraformaldehyde + 0.2% glutaraldehyde in 0.1 M PHEM buffer (60 mM Pipes, 25 mM Hepes, 2 mM MgCl₂ and 10 mM EGTA, pH 6.9) and then processed for ultrathin cryosectioning, 50 nm-thick cryosections were cut at −120°C using diamond knives in a cryo-ultramicrotome (Leica, Vienna) and transferred with a mixture of sucrose and methylcellulose onto formvar-coated copper grids. The grids were placed on 35-mm 2% gelatin plates at 37°C. The grids were incubated with the various antibodies at room temperature and then incubated with 10 nm protein A-conjugated colloidal gold (EM Laboratory, Utrecht, University). After immunolabeling, the sections were embedded in a mixture of methylcellulose and 0.6% uranylacetate and examined by a CM10 electron microscope (FEI, Eindhoven, the Netherlands) (Calafat et al., 1997).

Epon embedding

Cultured cells were fixed in Karnovsky fixative. To increase contrast, a post-fixation step with 1% Osmoniumtetroxide in 0.1 M cacodylate buffer was performed. After washing, the pellets were stained en bloc with Ultrastain 1 (Leica, Vienna, Austria) followed by an ethanol dehydration series. Finally, the cells were embedded in a mixture of DSDA/NMA-Embed-812 (EMS, Hatfield, U.S.A.), sectioned and stained with Ultrastain 1 and Ultrastain 2 (Leica, Vienna, Austria) before analysis by a CM10 electron microscope.

Ebola-VSV infection

Haploid wildtype Hap! cells (Carette et al., 2011) were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) supplemented with 8% FCS. Cells were electroporated with mRFP, mRFP-RILP or mRFP-RILP D125RE125 expression constructs. 24 hours post-transfection, the cells were exposed to AVSV-GP-EboV at MOI ~100 for 8 hours. 8 hours post-infection, cells were fixed with 4% formaldehyde and prepared for analysis by confocal microscopy or flow cytometry. 8 hours post-infection, cells were fixed with 4% formaldehyde in PBS for 30 minutes and mounted in Vectashield mounting medium (Vector Laboratories), as described above. The specimens were analyzed by confocal laser-scanning microscopes equipped with HCX Plan-Apochromat 63×1.2 NA and HCX Plan-Apochromat lbd.bl 63×NA 1.4 oil-corrected objective lenses (Leica). The acquisition software used was LCS (Leica). All images were taken with identical settings. For quantification, we selected transfected (marked by mRFP) cells and determined infection by AVSV-GP-EboV (marked by GFP expressed by the virus).

In order to visualize and quantify single and double fluorescent cells in our Ebola-VSV infection experiment (mask overlay), we used CellProfiler (http://www.cellprofiler.org) to detect and mask cells in the red and the green channel, which were subsequently overlaid to create the masked merged images. For flow cytometry analysis, the transfection and infection protocol was identical. Cells were harvested by trypsinization and fixed with 4% formaldehyde in PBS for 30 minutes. Cells were analyzed on a BD LSR II flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences) using appropriate settings to detect expressed by the virus).

For quantification, we selected transfected (marked by mRFP) cells and determined infection by AVSV-GP-EboV (marked by GFP expressed by the virus).

Statistical analysis

For calculation of correlation coefficients the signal intensity over a vector was plotted using the plot profile tool in ImageJ (http://imagej.nih.gov/ij/index.html) in both the green and red channel. Correlation coefficients for the two plots were calculated in Excel using the function:

\[
\text{Correl}(X,Y) = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sqrt{\sum (x-\bar{x})^2 \sum (y-\bar{y})^2}}
\]

For Fig. 3B, statistics were performed in Graphpad Prism using one-way ANOVA followed by Bonferroni’s post-hoc comparisons tests. All other statistics were performed in Microsoft Excel using a two sided Student’s t-test.

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Author contributions

R.v.d.K. designed and performed experiments, analysed data and wrote the paper; A.F. performed SPR experiments; L.I., S.K and N.N assisted with experiments; H.L. performed electron microscopy; T.B. and J.C. performed Ebola-VSV experiments; N.R. designed and performed experiments; and J.N. designed experiments and wrote the paper.

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References


antigen presentation, and microbial killing are controlled by the Arf-like GTase ArflB. Immunity 35, 182-193.


S1 C

GFP-tagged overexpressed construct

Endogenous

transfection efficiency 30-50%

S1 D

GFP-VPS33b

endogenous VPS33b

GFP-VPS33a

endogenous VPS33a

SPE-39-GFP

endogenous SPE-39

GFP-VPS16

endogenous VPS16

GFP-VPS11

endogenous VPS11
Fig. S1. RILP recruits VPS11, VPS41, VPS39, VPS16, VPS33b and SPE-39 to late endosomes. (A) Localization and membrane recruitment of HOPS complex subunits. MelJuSo cells expressing the GFP constructs indicated in combination with myc-RAB7Q67L or HA-RILP (as indicated) were fixed, immunolabelled with myc- or HA-antibodies and imaged by confocal fluorescence microscopy. Zoom-in indicated. Scale bar: 10μm. n>100 (B) Localization and endosomal membrane recruitment of VPS33a. MelJuSo cells expressing mRFP-VPS33a in the presence or absence of HA-RILP, GFP-VPS16 or combinations (as indicated) were fixed, immunolabelled with myc- or HA-antibodies and imaged by confocal fluorescence microscopy. Box indicate zoom-in area. Scale bar: 10μm. n>100. (C) Relative expression levels of GFP-tagged VPS proteins compared to their endogenous levels. MelJuSo cells were transfected with the constructs indicated and proteins were separated by SDS-PAGE and WB with antibodies indicated. Dark arrow shows position of GFP-tagged constructs, gray arrow the position of endogenous protein. Position of marker proteins indicated. (D) Tagged VPS proteins are incorporated into high molecular weight complexes. Lysates of MelJuSo cells expressing GFP-tagged VPS16, VPS11, VPS33b, VPS33a or SPE-39 were size separated by gel filtration. The elution of control proteins (and their mass) is indicated. Fractions of different sizes were separated by SDS-PAGE and WB and probed with antibodies indicated to detect complexes containing the GFP-tagged overexpressed proteins and their endogenous counterparts. For GFP-VPS33a only a very small fraction of total overexpressed protein was incorporated into higher molecular weight complexes, while other GFP tagged-HOPS subunits migrated similar as their untagged endogenous counterparts. MelJuSo cells expressing individual tagged-HOPS subunits (SPE39, VPS11, VPS18, VPS39, VPS41, VPS16, VPS33b) and HA-RILP were co-transfected with (E) mRFP-ORP1L (F) mRFP-ORP1LΔORD (G) mRFP-ORP1LΔORDFFAT(D478A). Cells were fixed and immunolabeled with HA-antibodies before imaging by confocal fluorescence microscopy. Boxed area shows the zoom-in. Scale bar: 10μm. Representative images for n>100.
S2 A

GAPDH

55 KDa
40 KDa
30 KDa

HA-RILP
HA-RILP ∆50
HA-RILP ∆100
HA-RILP ∆150
HA-RILP ∆199

RILP
RILP ∆50
RILP ∆100
RILP ∆150
RILP ∆199

CD63
merge
zoom

RILP
RILP ∆50
RILP ∆100
RILP ∆150
RILP ∆199

CD63
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RILP
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CD63
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Fig. S2. Binding of HOPS complex subunits to RILP. (A) Expression and localization of RILP truncation mutants. Left panel: HA-tagged RILP truncation mutants were expressed in MelJuSo cells. Lysates were separated by SDS-PAGE and WB and detected by anti-HA antibody. Expression of GAPDH is shown as a loading control. Marker proteins indicated. Right panel: MelJuSo cells expressing HA-tagged RILP truncation mutants were fixed, immuno-labeled with anti-HA and anti-CD63 antibodies and imaged by confocal microscopy. Box shows the zoomed-in area. Scale bar: 10μm. n>100. (B) Recruitment of VPS11, VPS39, VPS16, VPS33b and SPE-39 to N-terminal truncation mutants of RILP. MelJuSo cells expressing GFP-labelled HOPS subunits as indicated and HA-tagged truncation mutants of RILP (Δ50, Δ100, Δ150 and Δ199 respectively) were fixed, immunolabelled with HA-antibodies and imaged by confocal fluorescence microscopy. Box shows zoomed-in area. Scale bar: 10μm. n=100. (C) MelJuSo cells expressing GFP-VPS33a and HA-tagged truncation mutants of RILP (Δ50, Δ100, Δ150 and Δ199 respectively) were fixed, immunolabelled with HA-antibodies and imaged by confocal fluorescence microscopy. Box shows zoomed-in area. Scale bar: 10μm. n=100. Right panel: Quantification of VPS33a recruited to RILP mutants. Mean correlation coefficients ± sem are shown. n >10 for each condition. (D) Interaction of RILP truncation mutants with V5-VPS16. Lysates of Meljuso expressing V5-tagged VPS16 and GFP-RILP truncation mutants were immunoprecipitated (IP) with anti-GFP, then probed by anti-V5 and anti-GFP. TL: total lysate; IP: immunoprecipitation.
Fig. S3. **Proteins used in SPR-experiments.** (A) The N-terminus of VPS18 is sufficient for RILP recruitment. Upper panel: schematic map of VPS18 domains. Lower panel: MelJuSo cells expressing GFP-VPS18 [1-611] and RILP-HA were fixed, stained with anti-HA antibodies and imaged by confocal microscopy. The N-terminus of VPS18, as used for SPR experiments is recruited to RILP positive endosomes. Bar: 10 μm. (B) The Rab7Q67L/RILP complex is a stable complex. A Rab7Q67L complex with RILP was generated in the presence of GTP-γS and the temperature stability of the complex was measured. Red line is the melting curve in relative fluorescence units (RFU). The green line is first derivative of melting curve that shows Tm of 55°C (peak).
Fig. S4. RILP point mutants and the binding of HOPS subunits. Sequence alignment of the RILP N-terminus (Homo sapiens AA 1-200) and nine other species. Red: identical amino acids, dark green: conserved amino-acids, light green: similar amino acids. Asterisks indicate charged amino acids mutated in the opposite charge in our study. (B) Semi-quantitative representation of p150fli10 and HOPS subunit recruitment to RILP mutants (see Fig 4A, S4E-H). Levels of recruitment are depicted as ‘+’ (strong recruitment) to ‘-’ (no recruitment). (C) Expression levels of RILP point mutants expressed in MelJuSo cells. Cell lysates were separated by SDS-PAGE and WB probed with an anti-HA antibody. GAPDH is shown as a loading control. Marker proteins indicated. (D) MelJuSo cells expressing HA-tagged RILP point mutants were fixed, immuno-labeled with anti-HA and anti-CD63 antibodies and imaged by confocal microscopy. Boxed area zoomed-in. Scale bar: 10μm. Shown is representative cell of n>100. (E) RILP mutants and VPS39 and VPS18. MelJuSo cells expressing HA-RILP mutants (as indicated) and MYC-mRFP-VPS39 (left) or GFP-VPS18 (right) were fixed and immunolabeled with HA-antibodies and imaged by confocal fluorescence microscopy. Scale bar: 10μm. Representative of n>100 (F) RILP mutants and p150fli10 and VPS41. MelJuSo cells expressing GFP-RILP mutants (as indicated) were fixed and immunolabeled with p150fli10-antibodies (left). MelJuSo cells expressing HA-RILP mutants and GFP-VPS41 were fixed and immunolabeled with HA-antibodies and imaged by confocal fluorescence microscopy (right). Scale bar: 10μm. n>100 (G) RILP mutants, VPS11 and SPE-39. MelJuSo cells expressing HA-RILP mutants and GFP-VPS11 (left) or SPE-39-GFP (right) were fixed, immunolabeled with HA-antibodies and imaged by confocal fluorescence microscopy. Scale bar: 10μm. n>100 (H) RILP mutants, VPS33b and VPS16. MelJuSo cells expressing HA-RILP mutants and GFP-VPS33b (left) or GFP-VPS16 (right) were fixed, immunolabeled with HA-antibodies and imaged by confocal fluorescence microscopy. Scale bar: 10μm. n>100. (I) MelJuSo cells silenced for Dynein heavy chain and expressing HA-RILP199, HA-RILP D125R E126R or HA-RILP D125R E126R were fixed, immunolabeled with HA-antibodies and DAPI and imaged by CLSM. Scale bar: 10μm, representative of n>100. Right bottom panel: Quantification shows average number of individual vesicle clusters and/or free vesicles per cell. Cells with >10 clusters were set at 10 (means + SD; n>100). Right top panel: Western blot shows silencing for Dynein heavy chain by staining for co-depleted Dynein intermediate chain (Raaijmakers et al., 2012). (J) HAP-1 cells expressing mRFP, mRFP-RILP, mRFP-RILPD125R E126R or mRFP-RILPA199 were infected with RSVV-GP-EboV for 8 hours. After successful infection, the virus produces GFP in the host cell’s cytosol which is detected and quantified by flow cytometry. Shown is the percentage virus infection (by GFP) in mRFP-RILP or mRFP-RILP mutant expressing cells (mean ± sem). *p<0.05.
Table S1. Primers used in the study.