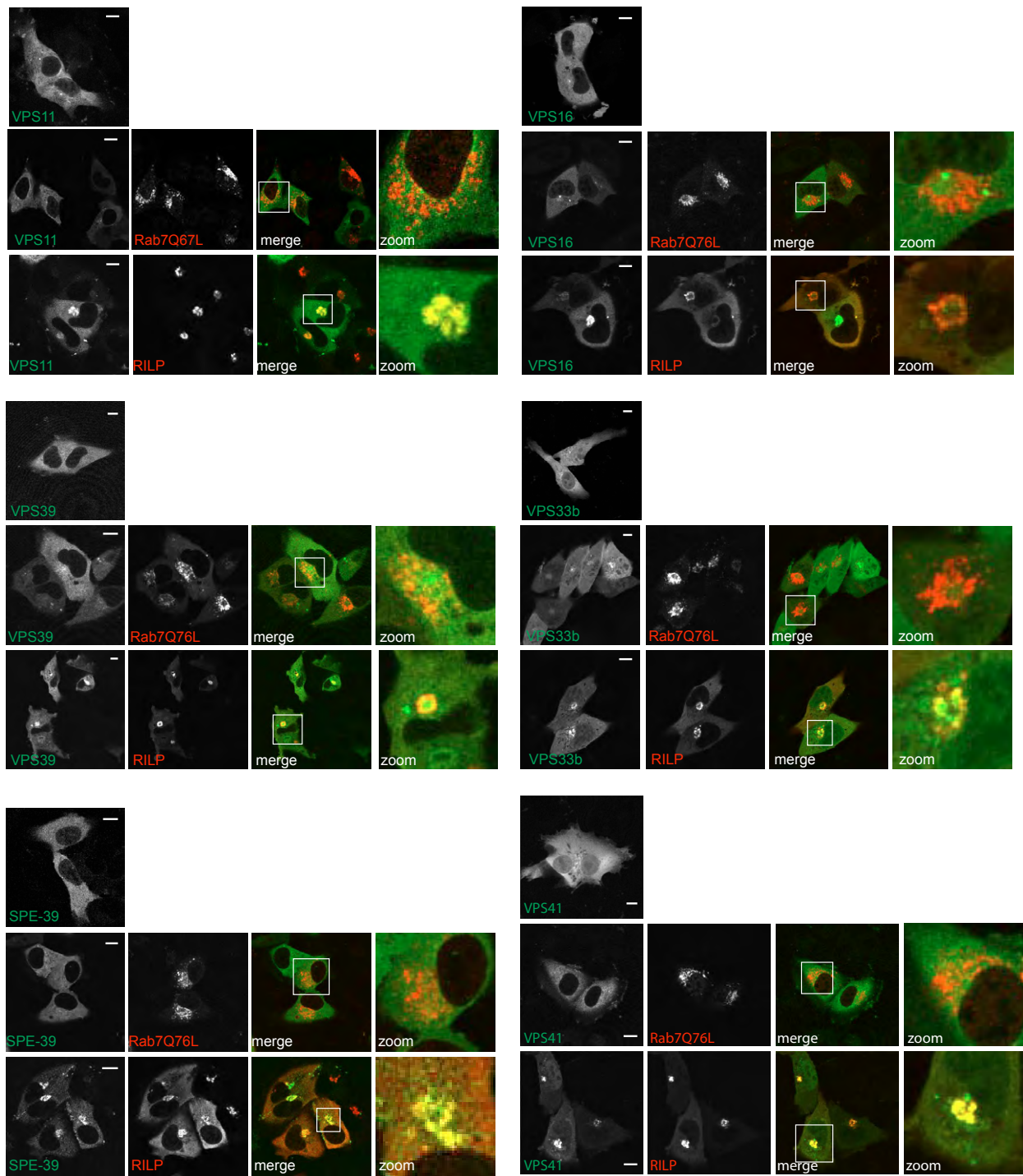
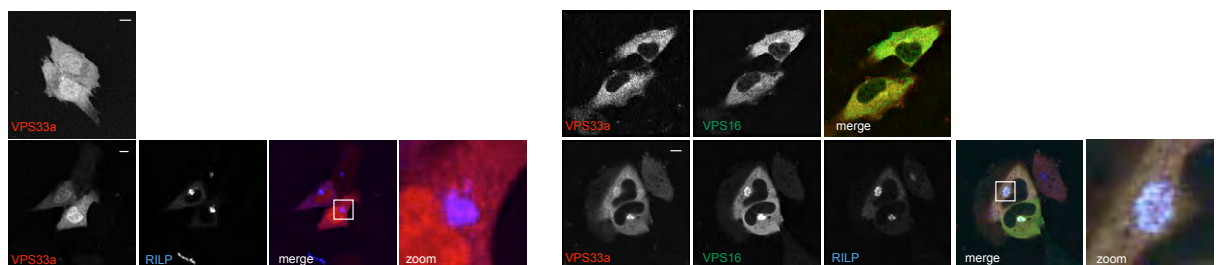


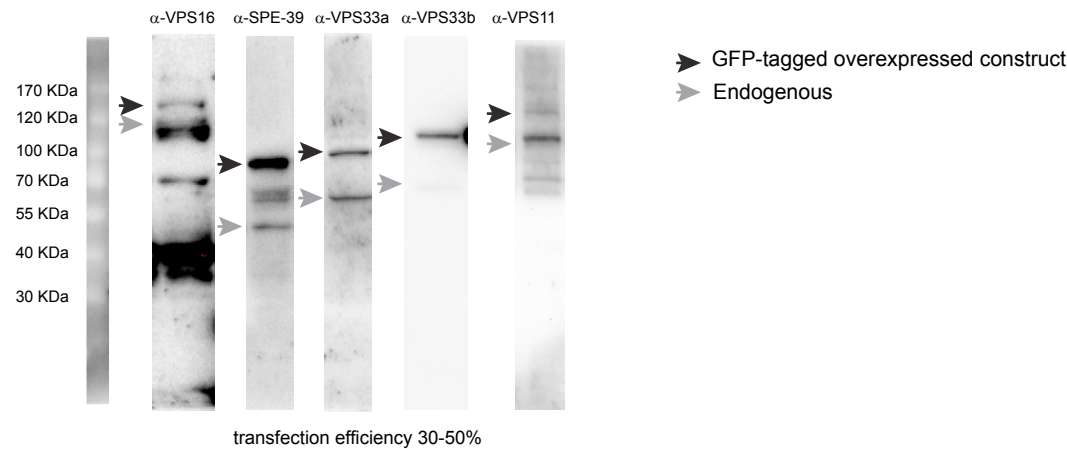
S1 A



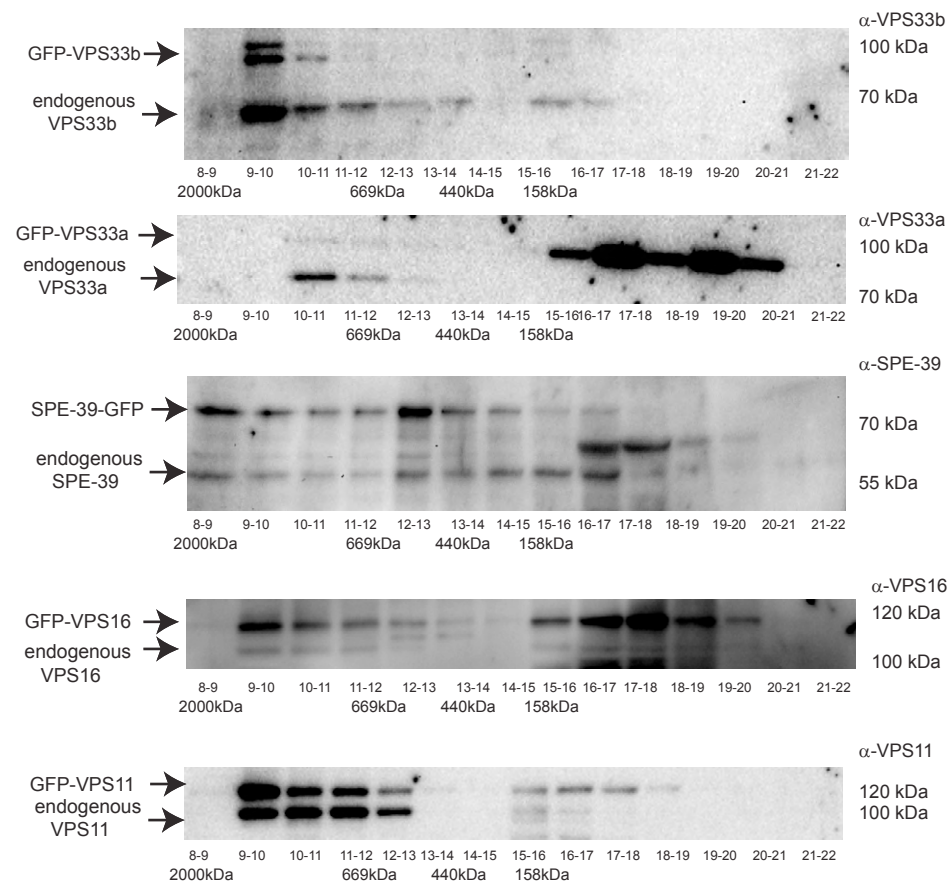
S1 B



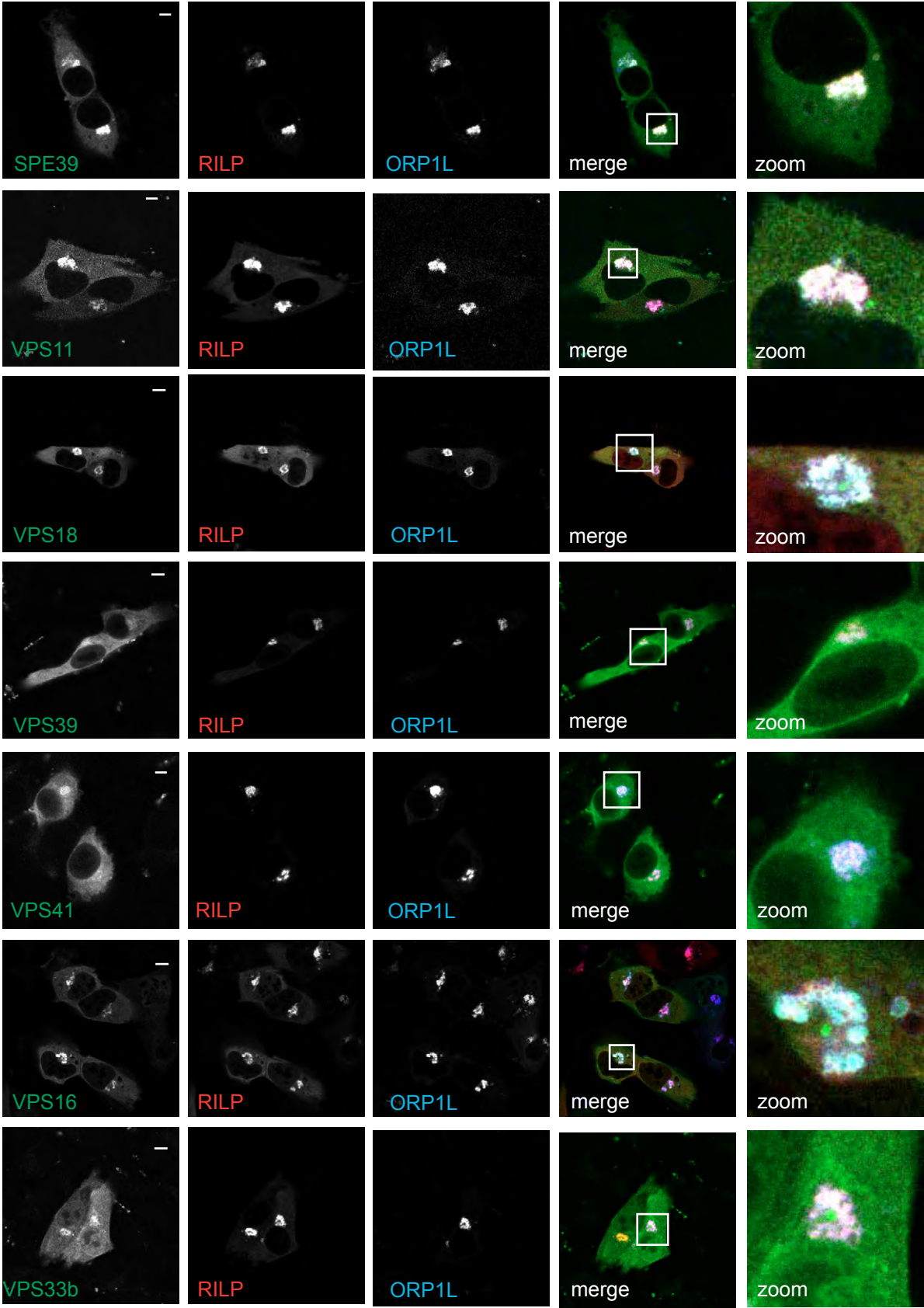
S1 C



S1 D

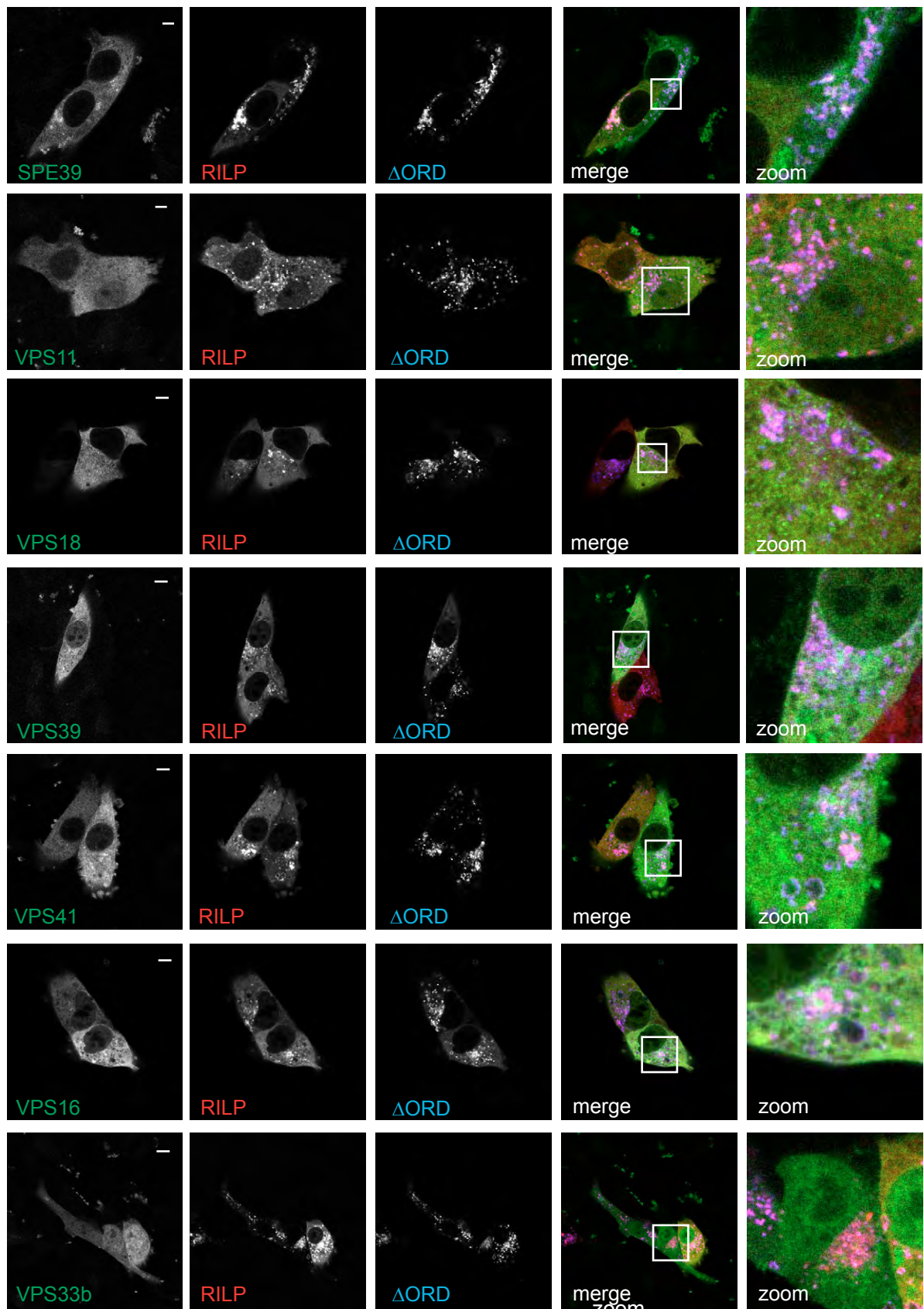


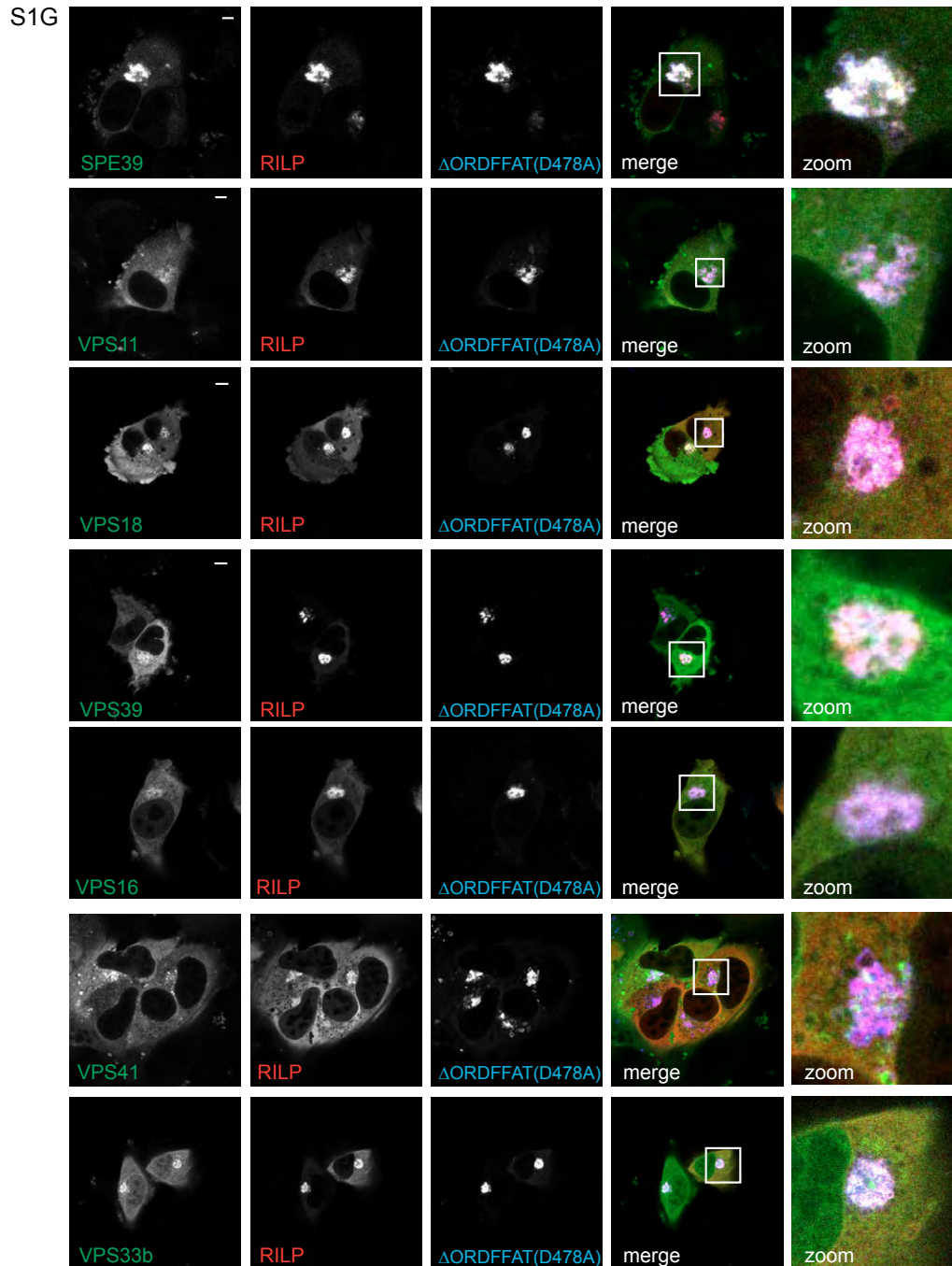
S1E





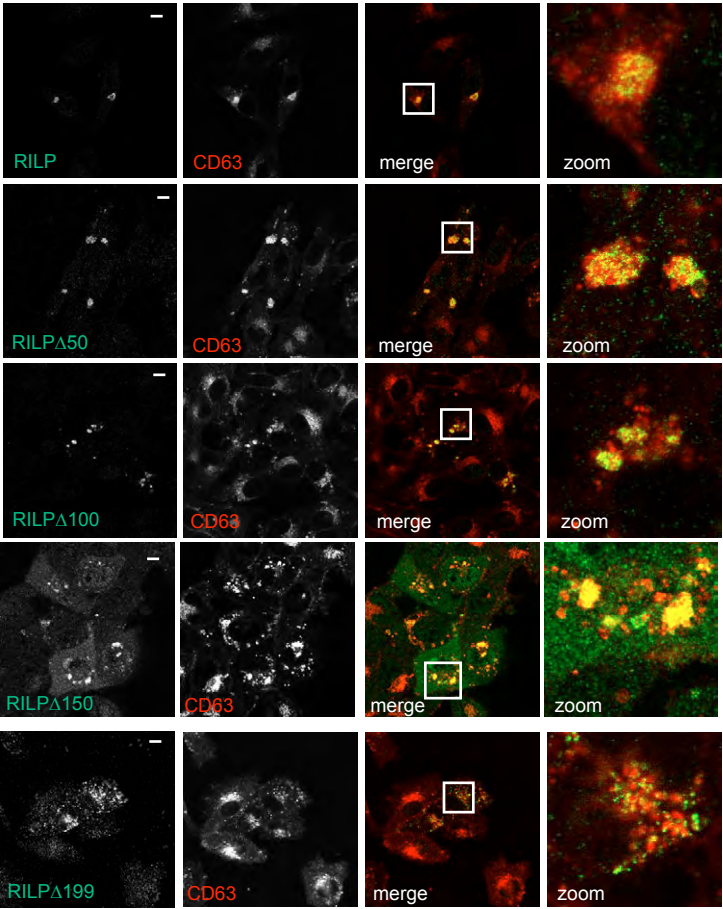
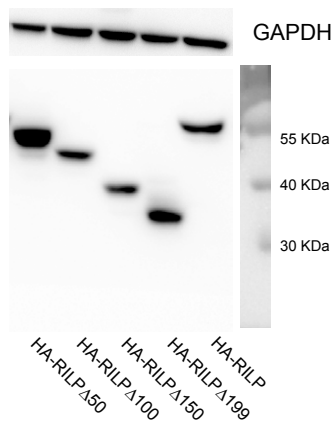
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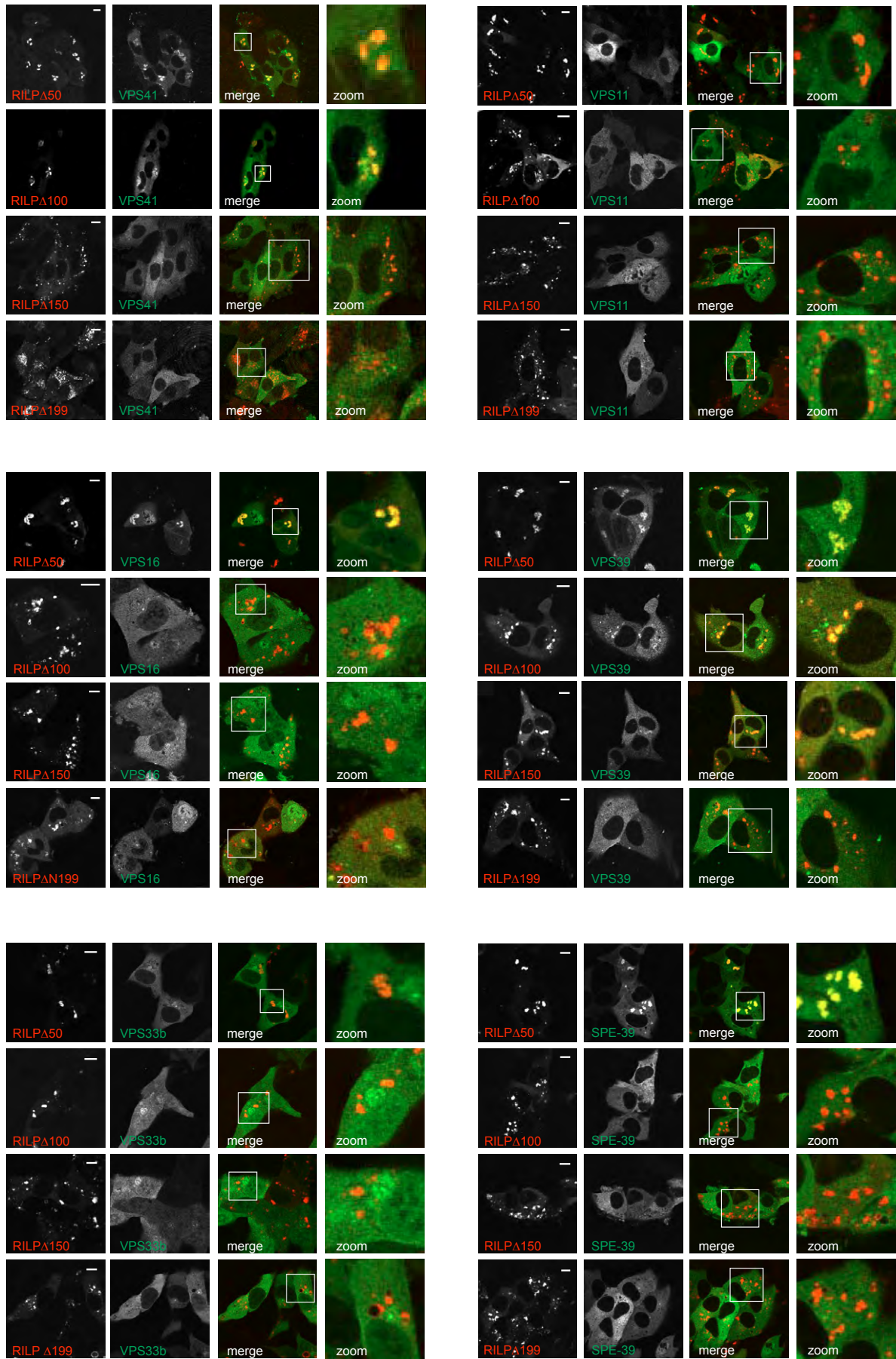
**Fig. S1. RILP recruits VPS11, VPS41, VPS39, VPS16, VPS33b and SPE-39 to late endosomes.** (A) Localization and membrane recruitment of HOPS complex subunits. MelJuSo cells expressing the GFP constructs indicated in combination with myc-RAB7Q67L or HA-RILP (as indicated) were fixed, immunolabelled with myc- or HA-antibodies and imaged by confocal fluorescence microscopy. Zoom-in indicated. Scale bar: 10 $\mu$ m. 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: 10 $\mu$ m. 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-ORP1L $\Delta$ ORD (G) mRFP-ORP1L $\Delta$ ORDFFAT(D478A). Cells were fixed and immunolabeled with HA-antibodies before imaging by confocal fluorescence microscopy. Boxed area shows the zoom-in. Scale bar: 10 $\mu$ m. Representative images for n>100.

S2 A

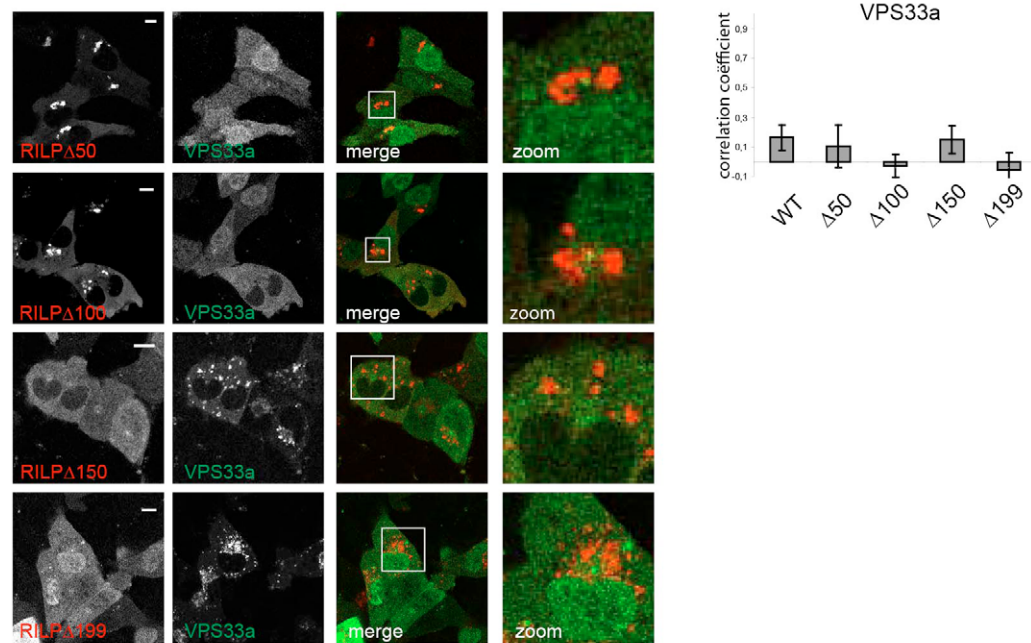




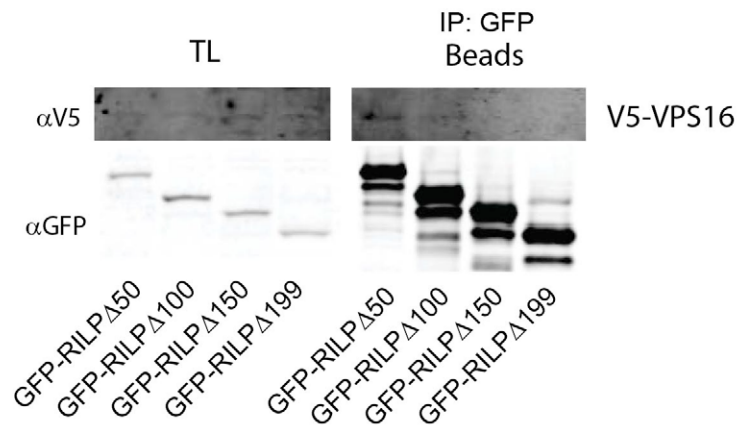
S2 B



## S2 C



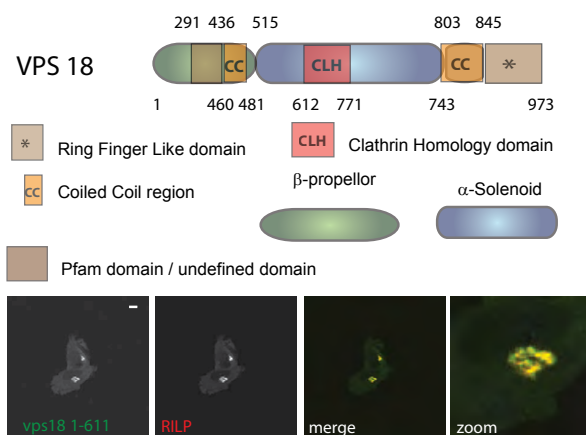
## S2 D



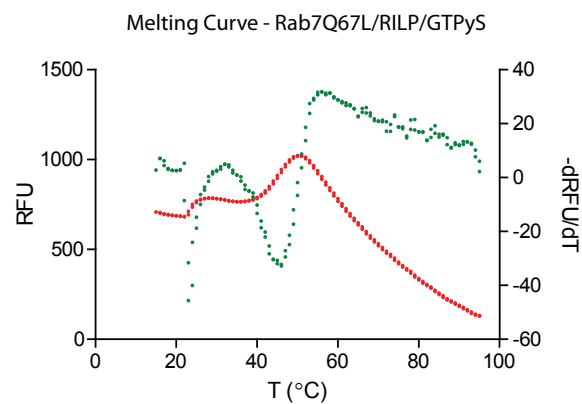
**Fig. S2. Binding of HOPS complex subunits to RILP.** (A) Expression and localization of RILP truncation mutants. Left panel: HA-tagged RILP truncation mutants were expressed in MelJuSo cells. Lysates were separated by SDS-PAGE and WB and detected by anti-HA antibody. Expression of GAPDH is shown as 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: 10 $\mu$ m. 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 ( $\Delta$ 50,  $\Delta$ 100,  $\Delta$ 150 and  $\Delta$ 199 respectively) were fixed, immunolabelled with HA-antibodies and imaged by confocal fluorescence microscopy. Box shows zoomed-in area. Scale bar: 10 $\mu$ m. n>100. (C) MelJuSo cells expressing GFP-VPS33a and HA-tagged truncation mutants of RILP ( $\Delta$ 50,  $\Delta$ 100,  $\Delta$ 150 and  $\Delta$ 199 respectively) were fixed, immunolabelled with HA-antibodies and imaged by confocal fluorescence microscopy. Box shows zoomed-in area. Scale bar: 10 $\mu$ m. 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.



S3 A

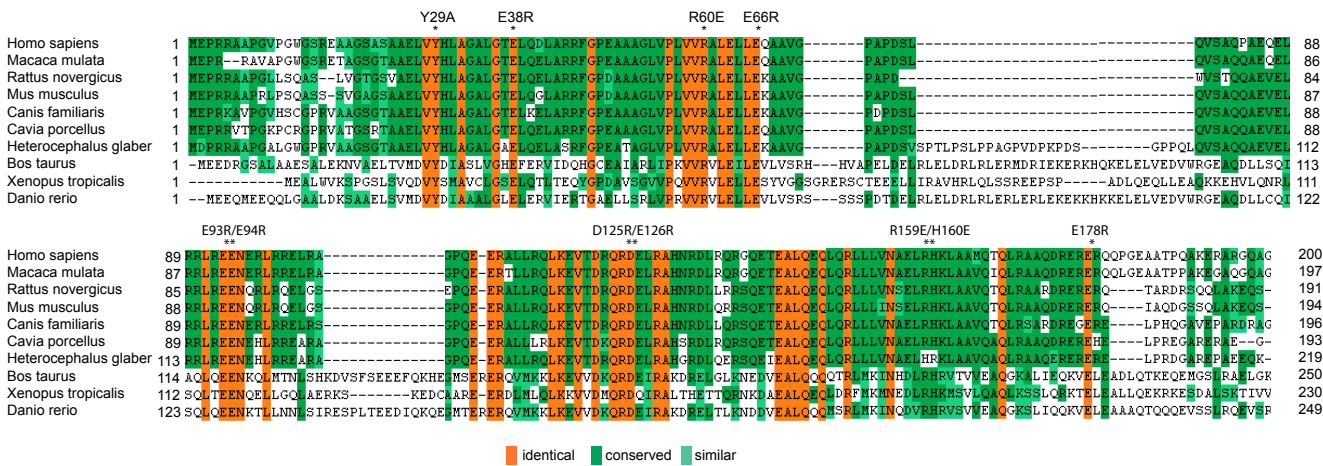


S3 B

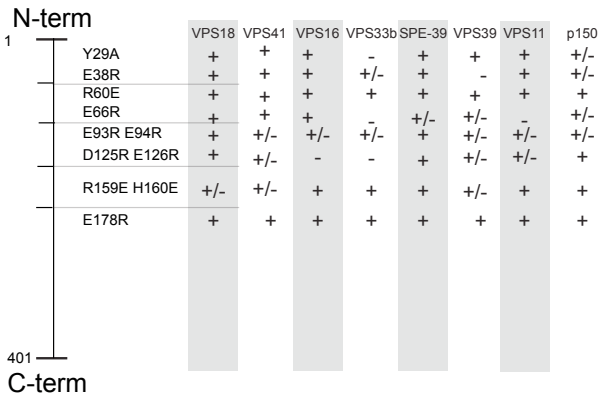


**Fig. S3. Proteins used in SPR-experiments.** (A) The N-terminus of VPS18 is sufficient for RILP recruitment. Upper panel: schematic map of VPS18 domains. Lower panel: MelJuSo cells expressing GFP-VPS18 [1-611] and RILP-HA were fixed, stained with anti-HA antibodies and imaged by confocal microscopy. The N-terminus of VPS18, as used for SPR experiments is recruited to RILP positive endosomes. Bar: 10  $\mu$ m (B) The Rab7Q67L/RILP complex is a stable complex. A Rab7Q67L complex with RILP was generated in the presence of GTP- $\gamma$ S 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  $T_m$  of 55°C (peak).

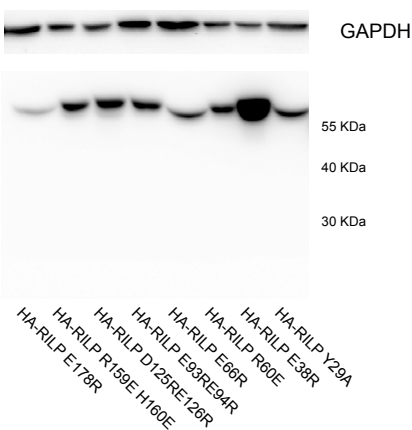
S4 A



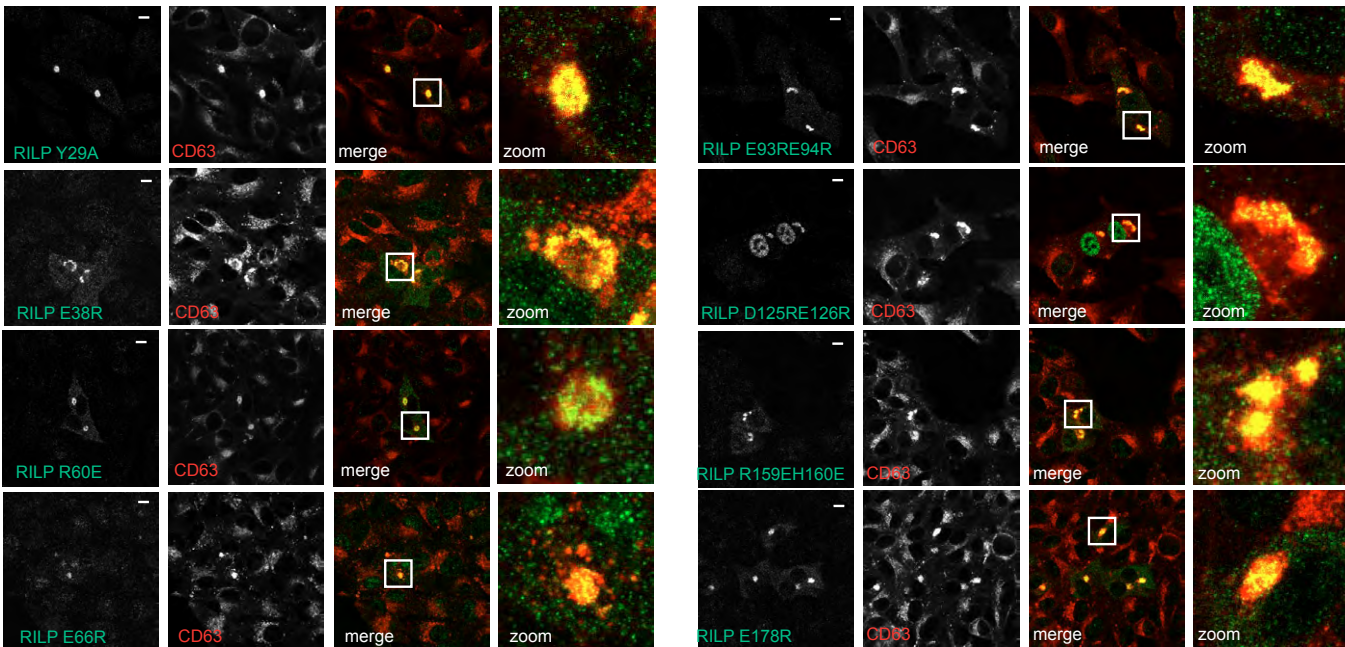
S4 B



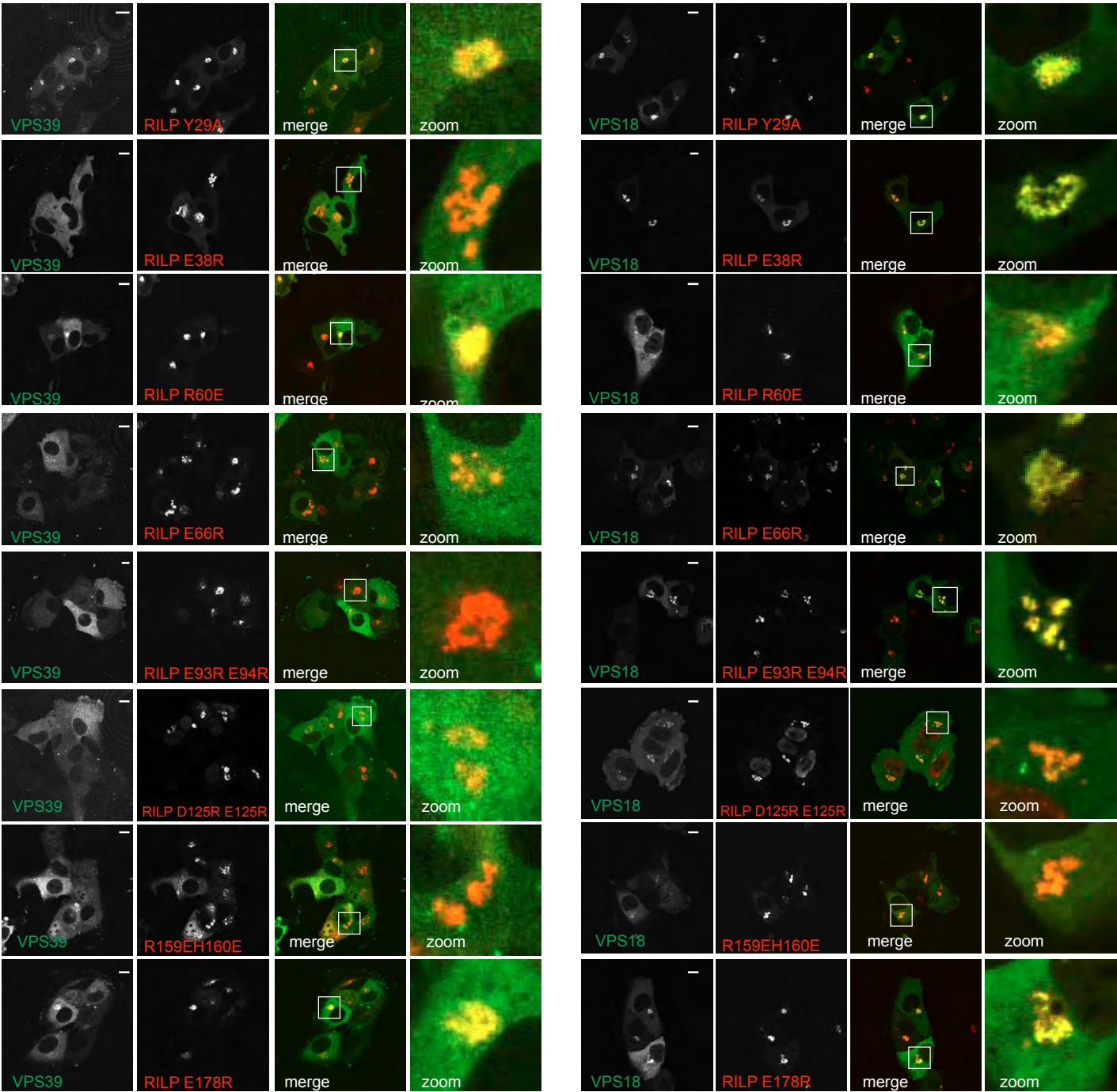
S4 C



S4 D

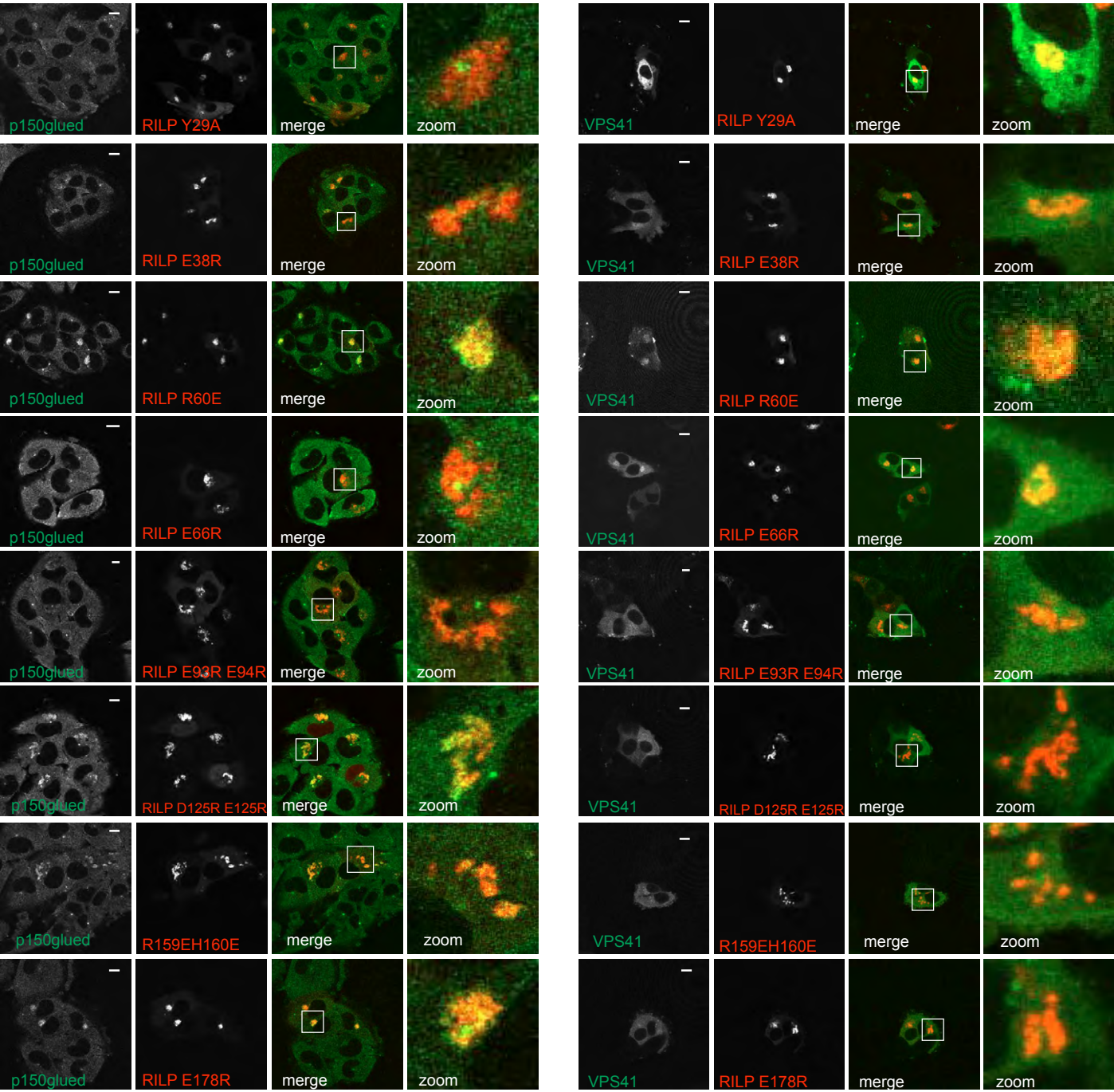


S4 E

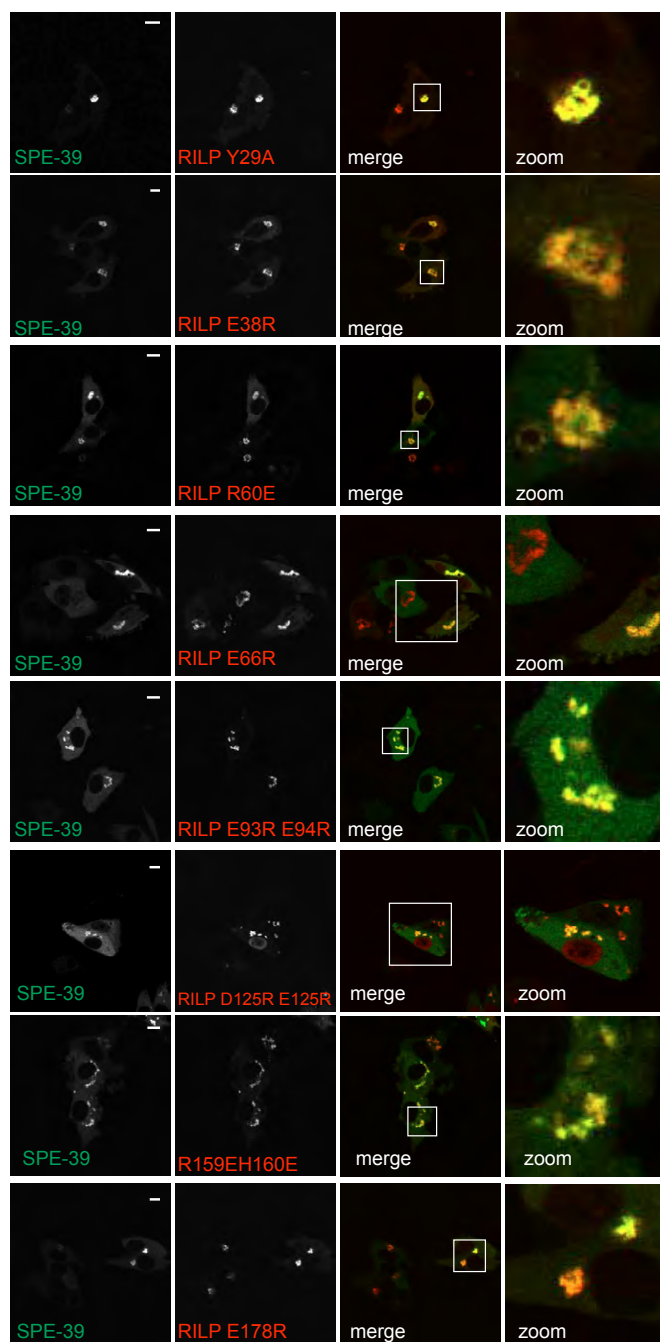
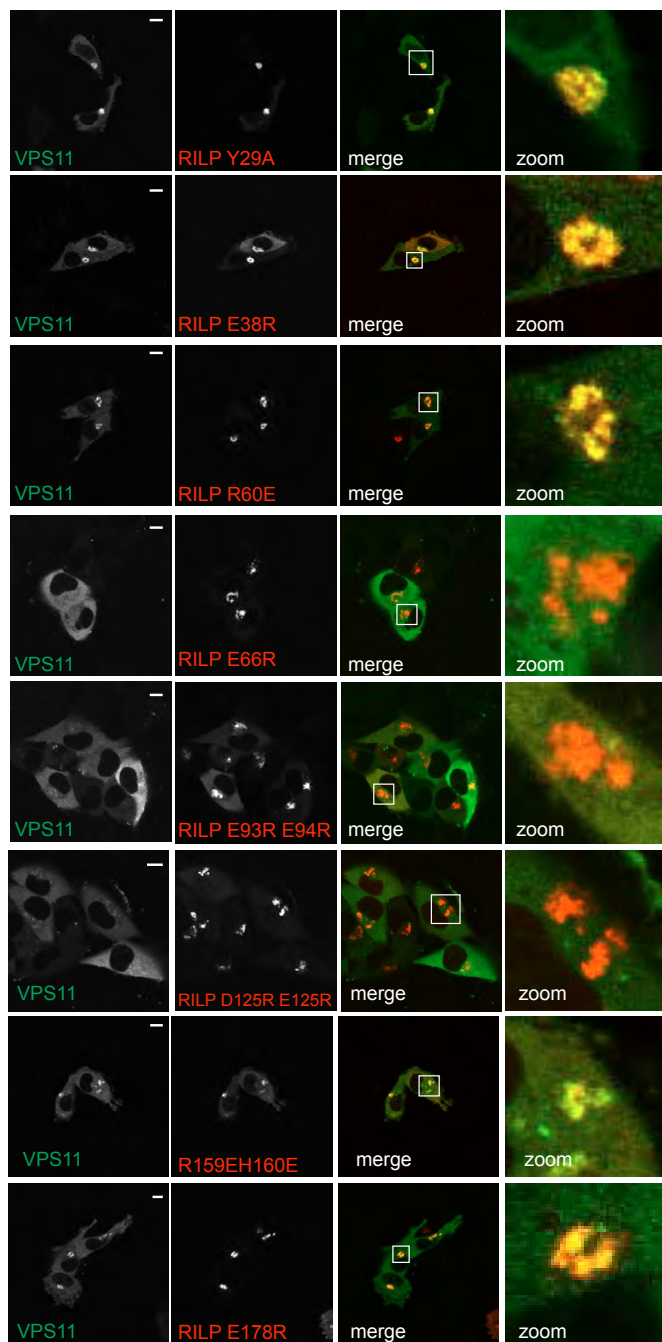




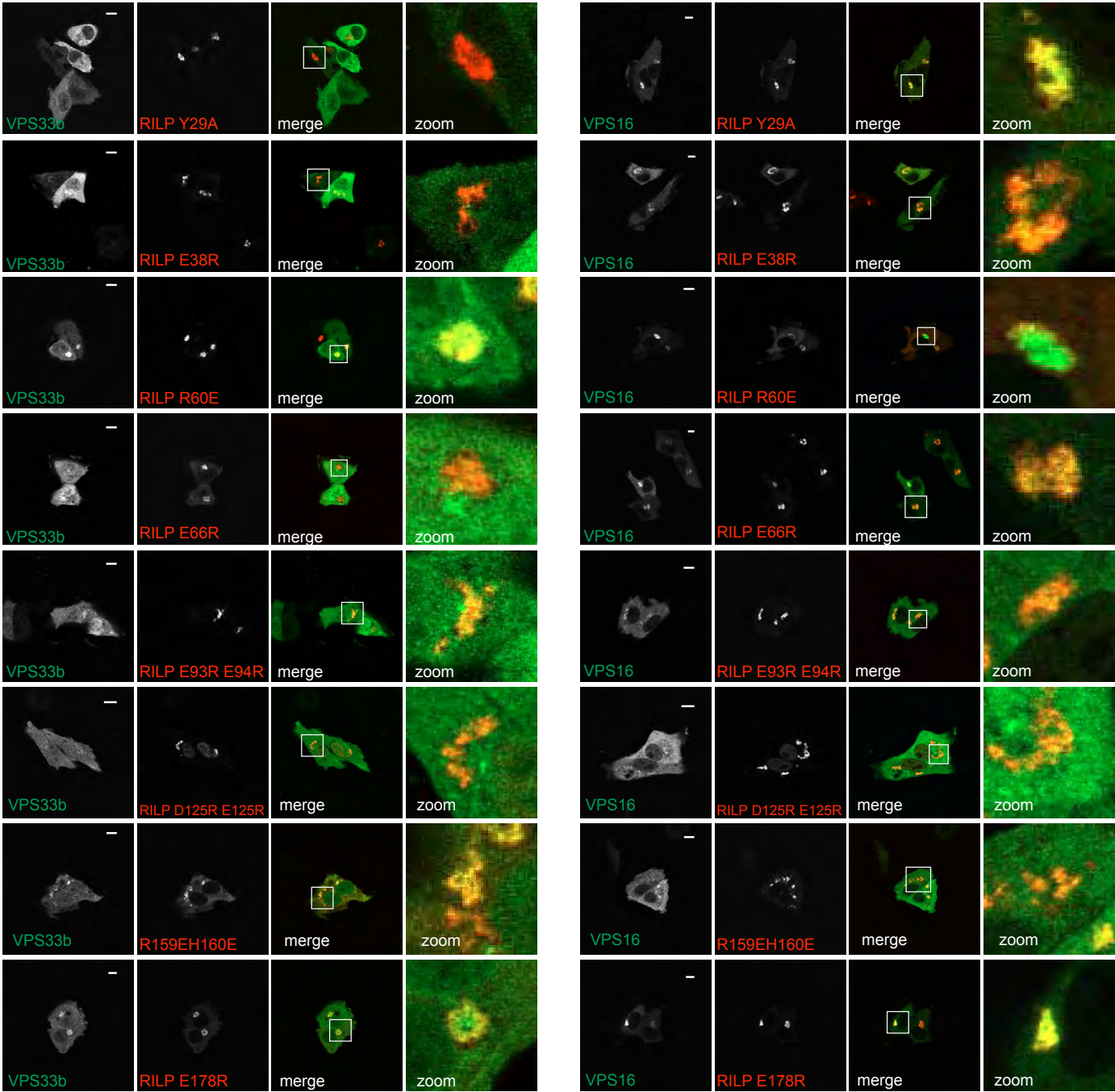
S4 F



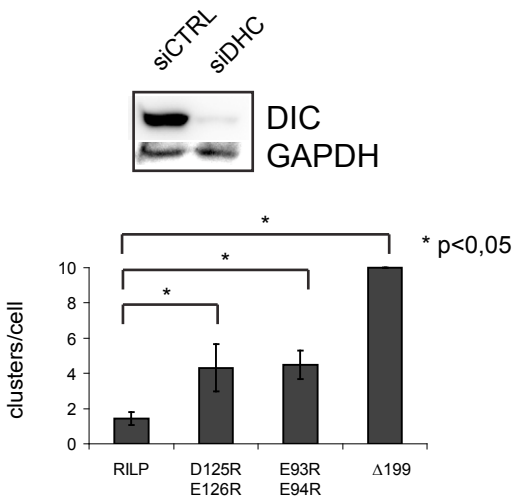
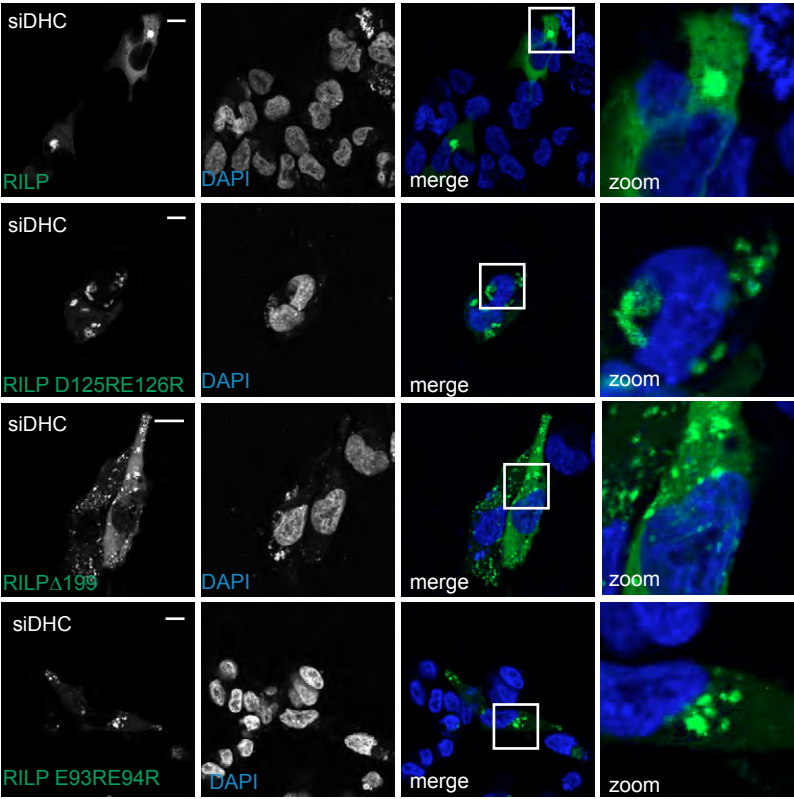
# S4 G

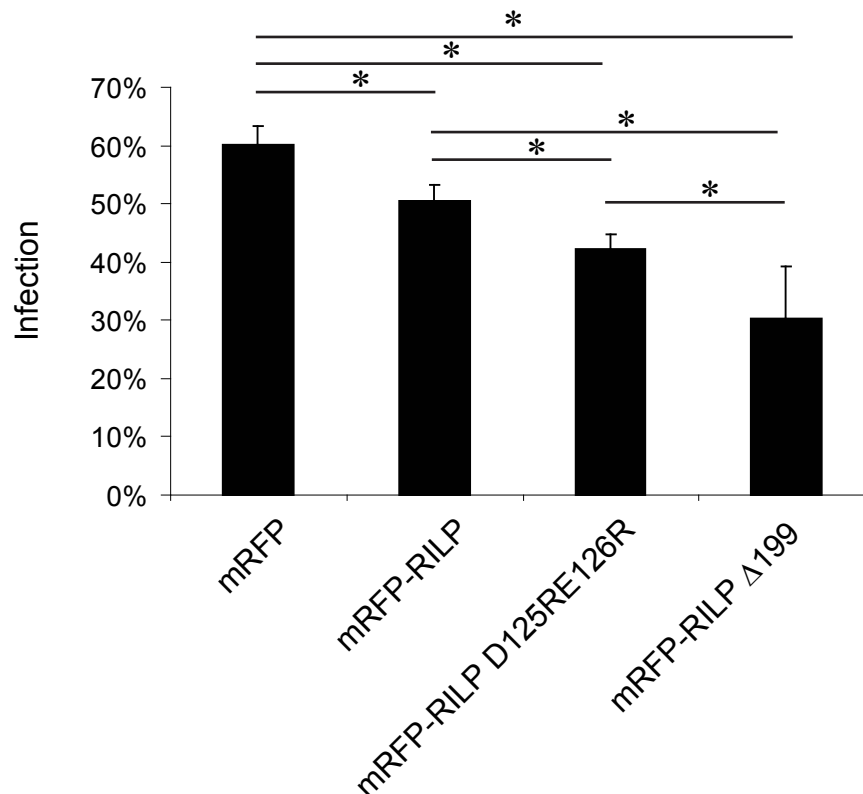


S4 H









**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 p150<sup>Glued</sup> 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 p150<sup>Glued</sup> and VPS41. MelJuSo cells expressing GFP-RILP mutants (as indicated) were fixed and immunolabelled with p150<sup>Glued</sup>-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-RILP, HA-RILPΔ199, HA-RILP D125RE126R or HA-RILP E93RE94R were fixed, immunolabelled 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-RILPD125RE126R or mRFP-RILPΔ199 were infected with rVSV-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.

	fwd	
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primerset 2	CCCAGAATTCCATGGCTTTTCCCCATCG	vps33b
primerset 3	CCCAGGATCCATGGCGGAAGCAGAGGAGCA	vps41
primerset 4	ATGGCGGCCTACCTGCAGTG	vps11
primerset 5	CCCAGAATTACCAACCATGAATCGGACAAAGGGTGA	spe39
primerset 6	CCCAGGATCCATGAATCGGACAAAGGGTGA	spe39
primerset 7	agaggAGGcgccagcagcctggcgaag	RILP E178R
		RILP
primerset 8	gctgGAGGAGaagctggcgccatgcagac	R159EH160E
		RILP
primerset 9	gcggCGCCGActccgggcgcaaccgc	D125RE126R
primerset 10	gcggAGGAGGaacgagcgctccgcagg	RILP
		E93RE94R
primerset 11	tcttgAGAcaggctgccgtggggccc	RILP E66R
primerset 12	tggtgGAGgcgctggagctcttgaaca	RILP R60E
primerset 13	agcgcCTCaccactagcggcaccagc	RILP E38R
primerset 14	cttgtgGCCcatctagccggggccctgg	RILP Y26A
	rev	
primerset 1	cccagaattcTACTTCTTCTGGGCTTGTG	vps16
primerset 2	CCCAGAATTCTCAGGCTTTTACCTCACTCA	vps33b
primerset 3	CCCAGGATCCCTATTTTTTCATCTCCAA	vps41
primerset 4	CCCAGAATTCTTAAGTGCCCC-TCCTGGAGT	vps11
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primerset 6	CCCAGAATTCTCAAGCGTAATCAGGAACGT	spe39
primerset 7	tggcgCCTcctctcgcggtcctgcgc	RILP E178R
		RILP
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primerset 9	ggagTCGGCGccgctgtcggtcctgacc	D125RE126R
primerset 10	cgttCCTCCTccgcagccgcccagctcc	RILP
		E93RE94R
primerset 11	gcctgTCTcaagagctccagcggcgc	RILP E66R
primerset 12	agcgcCTCaccactagcggcaccagc	RILP R60E
primerset 13	tgcagCCTagtggcggggcccgccg	RILP E38R
primerset 14	tagatgGGCcacaagctccgcccgat	RILP Y26A

**Table S1. Primers used in the study.**