Supplementary methods

In situ hybridization

In situ hybridization of third instar *Drosophila* wing discs with DIG-labelled RNA probes to *godzilla* (Roche, Basel) was performed according to protocol adapted from the Berkeley Drosophila Genome Project (BDGP) {Weiszmann, 2009 #2545}.

Supplementary Table Legend

Table S1. Raw data from UbiScan analysis.

Raw data of identified ubiquitylated VAMP3 peptides. Data from wild-type Godzilla is shown as CS12958 and CS12959, while ligase-dead is denoted as CS12960 and CS12961. This data is summarized schematically in Figure S6.

Supplementary Figure Legends

Figure S1. Godzilla ubiquitous expression in *Drosophila* wing discs.
(A,**B)** Anti-Godzilla antibody staining of *godzilla* mutant clone (**A**) or *MS1096-Gal4>UAS-godzilla* RNAi knock-down in third instar wing discs (B). **(C,D)** *in situ* hybridization of *godzilla* mRNA in control (**C**) or *godzilla* mutant wing discs (**D**). *godzilla* mRNA is widely expressed during embryogenesis and in third instar discs, here wing discs are shown as example. (E-G) Endogenous Godzilla protein is localized on endosomes in *Drosophila*. GFP-tagged endosome markers (Rab5, Rab7, or Rab11) were expressed with the salivary gland driver AB1-Gal4 (E) or the wing disc driver MS1096-Gal4 (F). Tissues were stained with anti-Godzilla antibody (red) and phalloidin (blue). (G) Endogenous endosome marker proteins were stained with specific antibodies together with anti-Godzilla.

Figure S2. Expression of Goliath and Godzilla in HEK293 cells.
Detergent-solubilized cell lysates of Goliath-C-GFP, Godzilla-C-GFP, or pCGFP (mock) transfected cells were analyzed by immunoblotting. Anti-GFP antibody was used to visualize proteins. The expression level of both proteins is comparable and no significant degradation is observed. The relative molecular weight of each protein is higher than predicted from amino acid sequence, suggesting a post-transcriptional modification such as glycosylation or ubiquitylation.

Figure S3. Endosomal localization of Goliath-HA and Godzilla-HA.
HA-tagged proteins were expressed in HEK293 cells and cells were stained with anti-EEA1 or M6PR antibody. Similar to the results with GFP-tagged Goliath and Godzilla proteins (Figure 2A), both Goliath-HA and Godzilla-HA localize on EEA1 positive endosomes, and their expression leads to the accumulation of enlarged EEA1 positive endosomes.

Figure S4. Godzilla expression induces apoptosis in *Drosophila*.
GFP-tagged Godzilla (*green*) was ectopically expressed by *MS1096*, a wing disc driver. Cell shape is visualized with phalloidin to access the integrity of

the epithelium (F-actin; *red*).

Figure S5. Generation and genomic rescue of Godzilla mutants.

(A) Schematic drawing of genomic DNA surrounding the *godzilla* locus. *Δgodzilla*^{1} was generated from two *Exelixis* mutant (XPd01485 and WHf07224). *Δgodzilla*^{2} was generated by imprecise P-element excision of EP705. Genomic rescue for *godzilla* mutant (*Δgodzilla*^{2} · *godzilla*) was generated by using ϕ31 integrase-mediated fosmid transgene based on *Δgodzilla*^{2}. **(B)** The giant endosome phenotype of *godzilla* mutant is significantly rescued by *godzilla* genomic rescue.

Figure S6. Summary overview of UbiScan ubiquitylation of VAMP3.

(A) Schematic overview of ubiquitylation sites identified on VAMP3. In the absence of Godzilla activity (left), VAMP3 is mainly ubiquitylated at two Lys residues in the coiled-coil region (Lys^{35} and Lys^{42}), while three Lys residues in the linker region are highly ubiquitylated in the presence of Godzilla (right). **(B)** Summary overview of UbiScan data for all seven Lys residues present in VAMP3. Degree of ubiquitylation at each Lys residue is indicated by the number of *plus* (+ to +++). *Minus* - no ubiquitylation has been identified or reported at corresponding sites. The quantitative difference in ubiquitylation between wild-type Godzilla and ligase-dead is shown as *Fold change*. Raw data is supplied in Supplementary Table 1. For reference, numbers of the publicly available identified ubiquitylated sites from previous CST studies are displayed in the right column (*data from PhosphoSitePlus, http://www.phosphosite.org/).

Figure S7. Rab11 recycling endosomes are significantly reduced in Godzilla overexpressing tissues in *Drosophila*.

UAS-GFP^{CAAX} (*top*) or *UAS-Godzilla-GFP* (*bottom*) was expressed in *Drosophila* wing disc with *MS1096-Gal4*. Rab11 was visualized by anti-Rab11 antibody (*red*). Significant loss of Rab11 positive vesicles is observed upon *UAS-Godzilla-GFP* expression (*red in bottom panel*).

Figure S8. siRNA-mediated knockdown of VAMP3 in HEK293 cells.

The efficiency of siRNA-mediated knockdown in HEK293 cells was confirmed by immunoblotting and immunofluorescence. Knockdown of endogenous VAMP3 reduces Rab11 recycling endosomes, but does not affect localization or vesicle size of EEA1 endosomes.

Figure S1

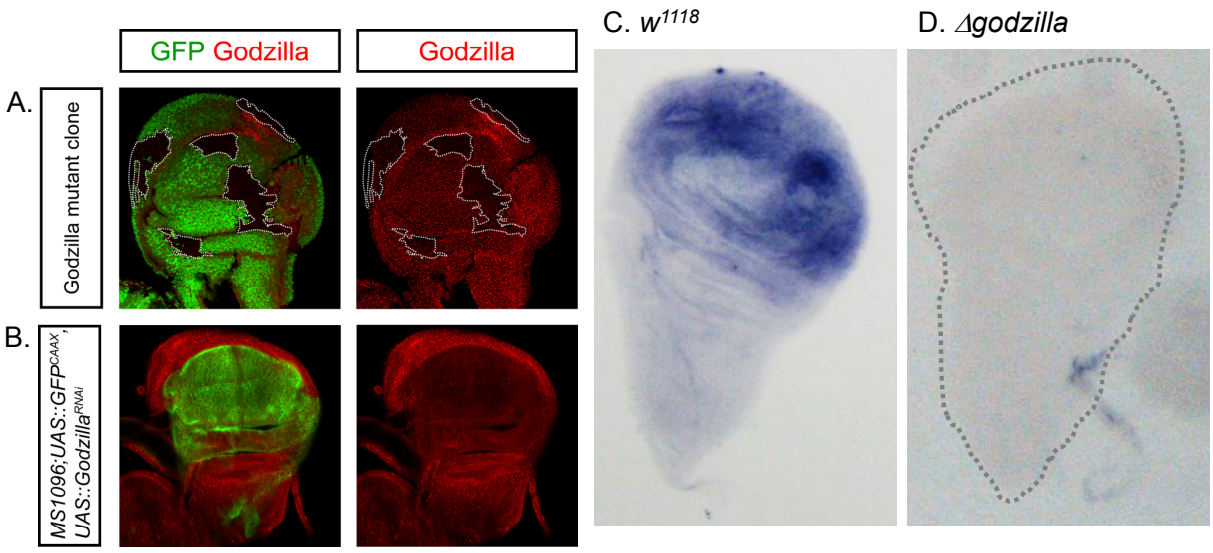

GFP Godzilla
Godzilla
A.
Godzilla mutant clone
B.
MS1096;UAS::GFP^CAAX,
UAS::Godzilla^RNAi
C. w^1118
D. Δgodzilla

Figure S1

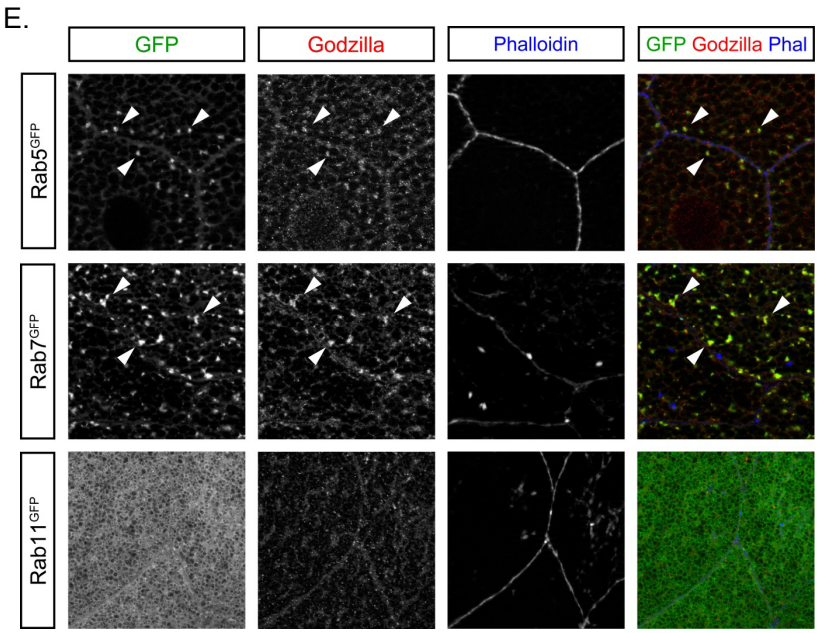

E.
GFP
Godzilla
Phalloidin
GFP Godzilla Phal
Rab5GFP
Rab7GFP
Rab11GFP

Figure S1

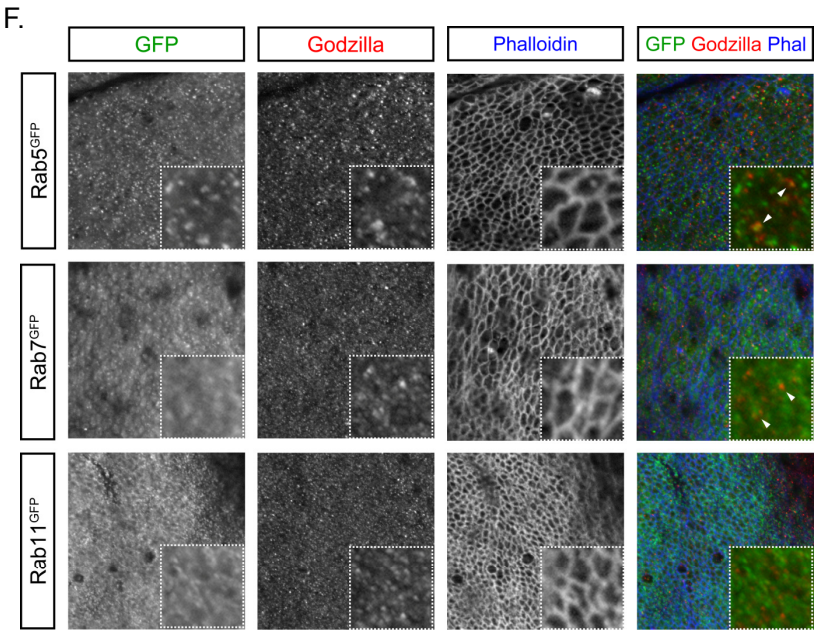

F.
GFP
Godzilla
Phalloidin
GFP Godzilla Phal
Rab5GFP
Rab7GFP
Rab11GFP

Figure S1

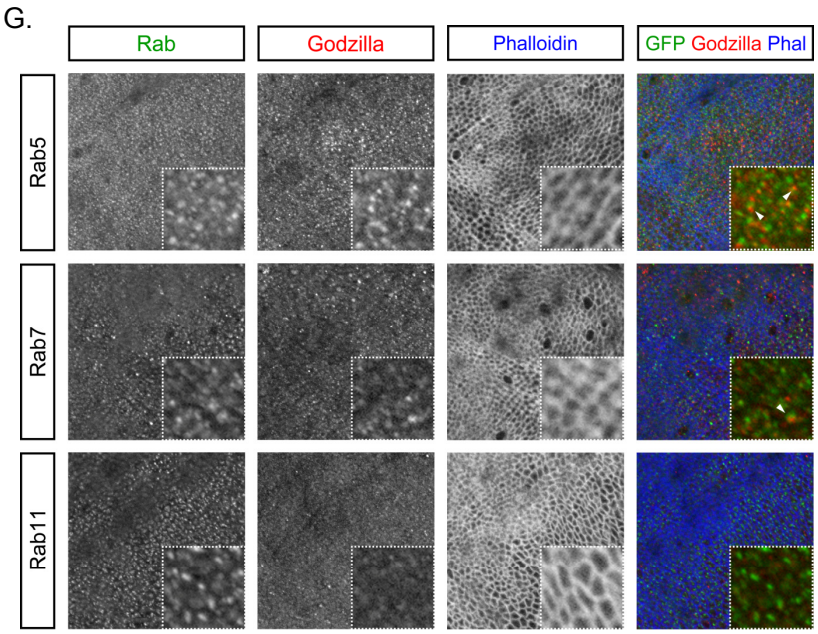

G.
Rab
Godzilla
Phalloidin
GFP Godzilla Phal
Rab5
Rab7
Rab11

Figure S2

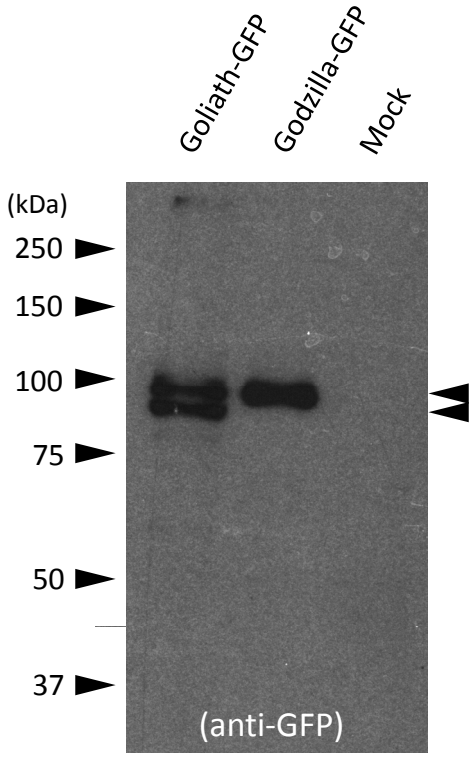

Goliath-GFP
Godzilla-GFP
Mock
(kDa)
250
150
100
75
50
37
(anti-GFP)

Figure S3

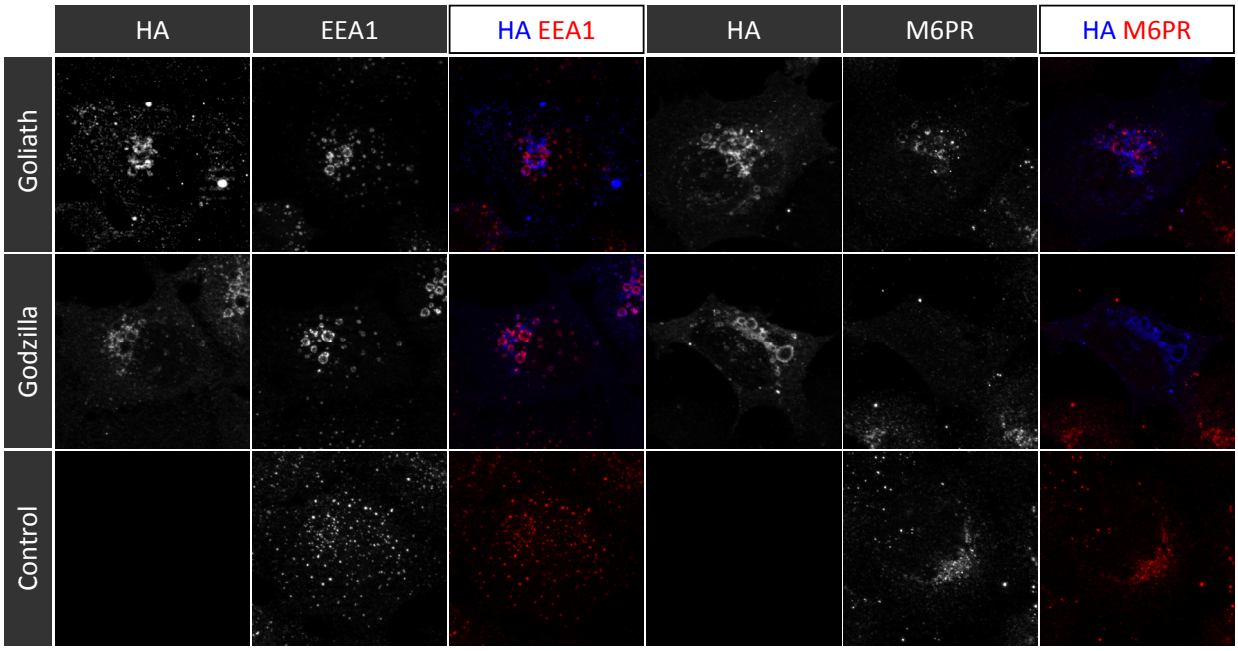


Figure S4

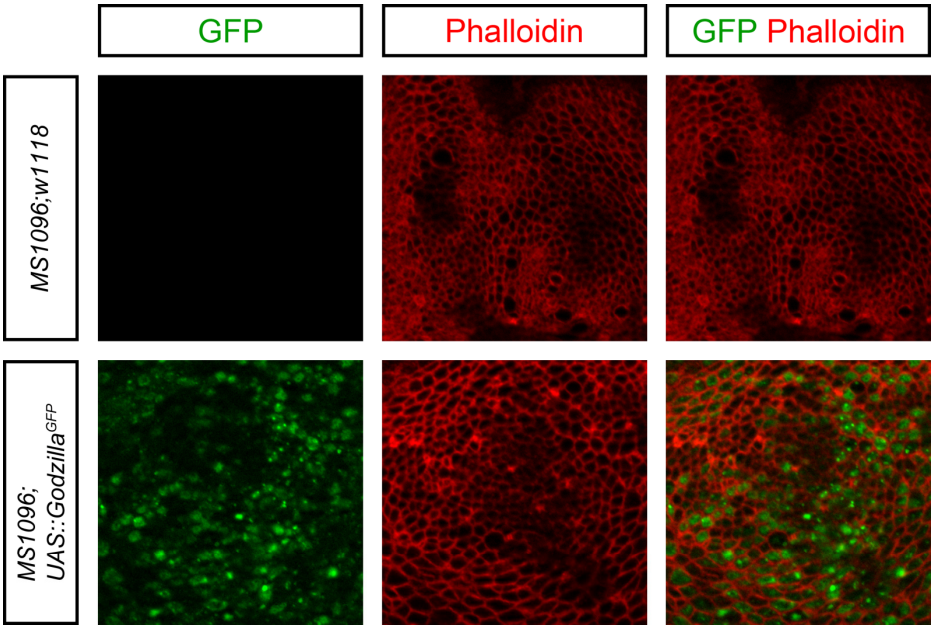

GFP
Phalloidin
GFP Phalloidin
MS1096;w1118
MS1096;
UAS::Godzilla^GFP

Figure S5

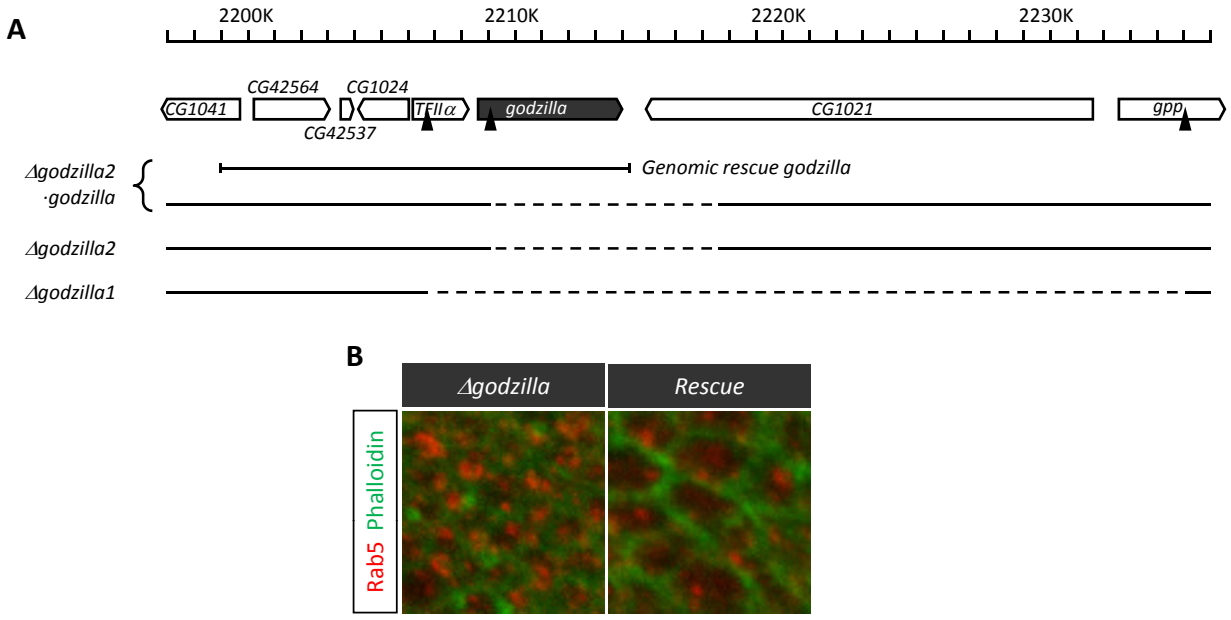
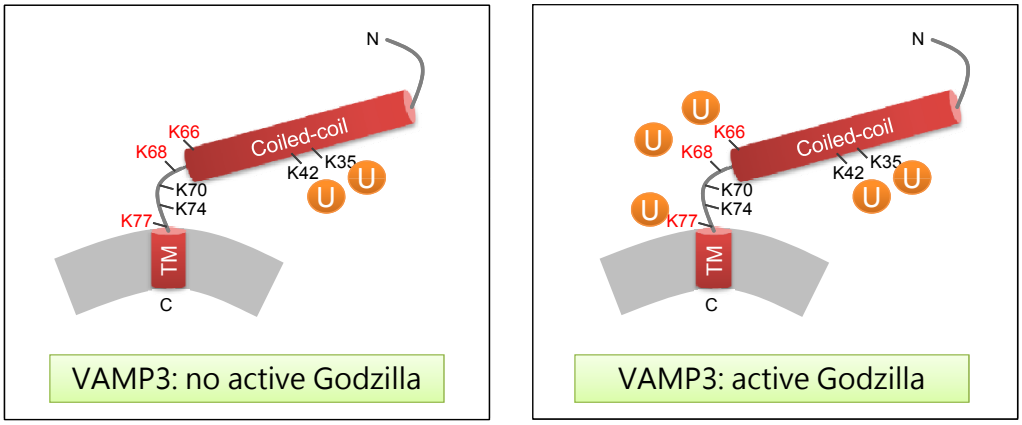

A
2200K
2210K
2220K
2230K
CG1041
CG42564
CG42537
CG1024
TFIIα
godzilla
CG1021
gpp
Δgodzilla2
·godzilla
Genomic rescue godzilla
Δgodzilla2
Δgodzilla1
B
Δgodzilla
Rescue
Rab5 Phalloidin

Figure S6

A



B

	HEK293 transfected with Godzilla ligase-dead	HEK293 transfected with WT Godzilla	Fold change	PhosphoSitePlus data*
K35	+++	+++	2.3	90
K42	++	+++	3.0	88
K66	+	+++	21.1	15
K68	+	+++	15.4	5
K70	-	-	-	-
K74	-	-	-	-
K77	-	++	53.1	-

Figure S7

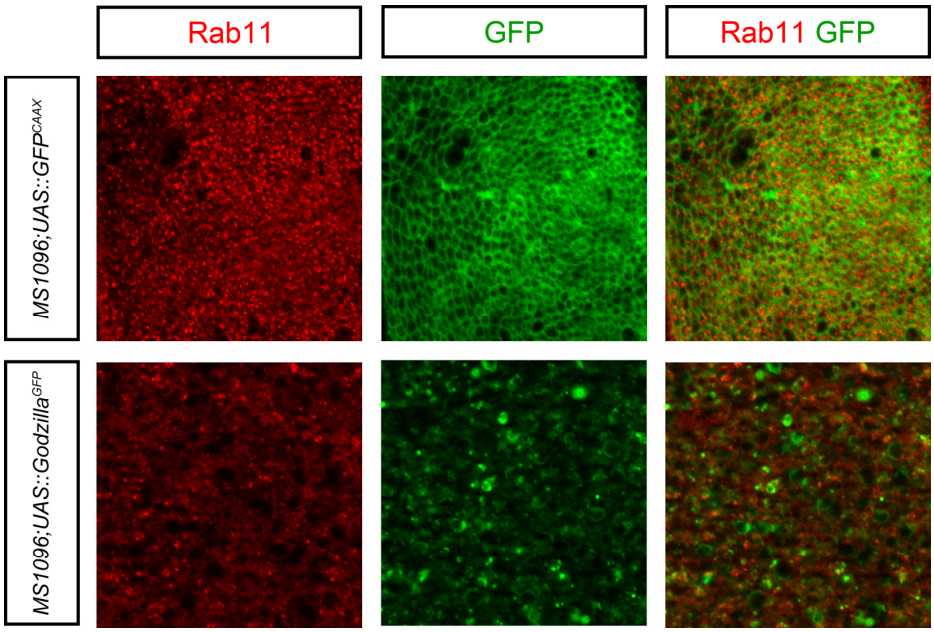
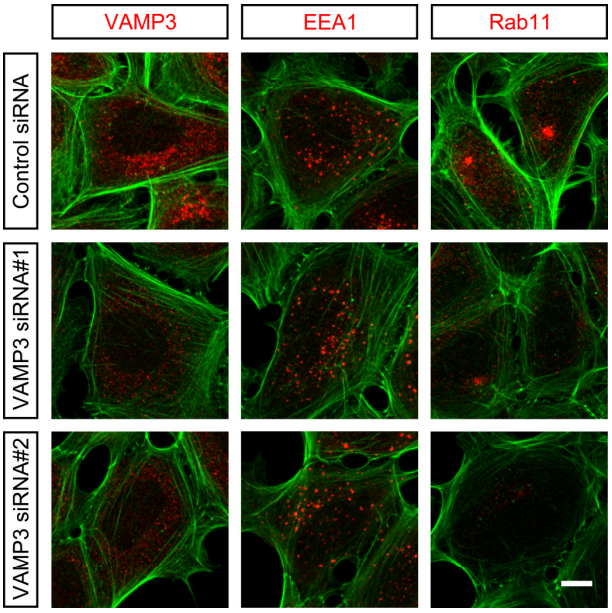
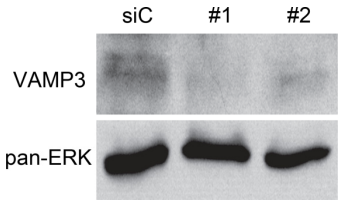

Rab11
GFP
Rab11 GFP
MS1096;UAS::GFPCAAX
MS1096;UAS::GodzillaGFP

Figure S8


VAMP3
EEA1
Rab11
Control siRNA
VAMP3 siRNA#1
VAMP3 siRNA#2


siC
#1
#2
VAMP3
pan-ERK