IN VITRO BUDDING OF INTRALUMENAL VESICLES INTO LATE ENDOSOMES IS REGULATED BY ALIX AND TSG101 [Thomas Falguières, Pierre-Philippe Luyet, Christin Bissig, Cameron C. Scott, Marie-Claire Velluz and Jean Gruenberg]

LEGENDS OF SUPPLEMENTARY FIGURES

Figure S1. HPTS quenching with DPX. (A) In the test tube, 1mM HPTS was incubated with the indicated concentrations of DPX. Remaining fluorescence was measured and expressed as a percentage of total fluorescence without DPX quenching. Error bars are too small to be visible. **(B-C)** The in vitro assay measuring HPTS incorporation was performed using PNS (protocol 1) or purified late endosomes (protocol 2), as in Figure 1. HPTS fluorescence emission was then quantified and typical examples of the raw values are indicated. Using protocol 1, excess HPTS is removed during fractionation, and thus the background is much lower than with protocol 2.

Figure S2. Time-course of HPTS incorporation into endosomes labeled with endocytosed BSA-Cy3. BHK cells were incubated with 0.1mg/ml BSA-Cy3 for 90min at 37°C as in Figure 2A, and then PNS were prepared and used in the in vitro assay with protocol 1 (Figure 1A) for the indicated time period. Then, isolated late endosome fractions were analyzed by confocal microscopy as in Figure 2B. Fluorescence of BSA-Cy3 (red – top panels) and HPTS (green – middle panels) were merged (bottom panels). Arrowheads point at examples of structures positive for both HPTS and BSA-Cy3. Bars: 20µm.

Figure S3. EGF and EGF receptor transport in BHK cells overexpressing the human EGF receptor. (A) BHK cells expressing GFP-tagged human EGF receptor under the control

of a tetracycline promoter were treated (+) or not (-) with 1μg/ml doxycycline for 24h. After cell lysis, samples were processed for western blot using an anti-GFP monoclonal antibody. **(B)** After induction (bottom panels) or not (upper panels), as in (A), followed by serum-starvation, cells were incubated on ice with anti-EGF receptor antibody followed by Cy5-coupled anti-mouse antibody (blue), and Texas Red-coupled EGF (red). Then cells were incubated for 50 min at 37°C, fixed and processed for indirect immunofluorescence against Lamp1, using an AMCA-coupled secondary antibody (purple), as in Figure 3D. Note that in non-treated cells, EGF receptor-GFP, anti-EGF receptor and EGF signal intensities are very weak in comparison to doxycycline-treated cells. Bars: 50 μm.

Figure S4. Time-dependence of HPTS incorporation. The time-dependence of HPTS incorporation in late endosomes was measured in vitro using protocol 1 (A) and protocol 2 (B). The means (±SEM) of three independent experiments are shown.

Figure S5. HPTS incorporation after endosome pre-incubation. (A) The outline shows the protocol that was used to pre-incubate membranes and cytosols before the assay. As indicated, late endosomes and cytosols were incubated together or separately at 4°C or 37°C for 20min. Then HPTS was added as indicated and the mixtures were incubated for 20min at 37°C, using protocol 2 (Figure 1A). **(B)** The assay was carried out as outlined in (A). HPTS fluorescence is expressed as a percentage of the control (protocol a in A). The means (±SEM) of three independent experiments are shown.

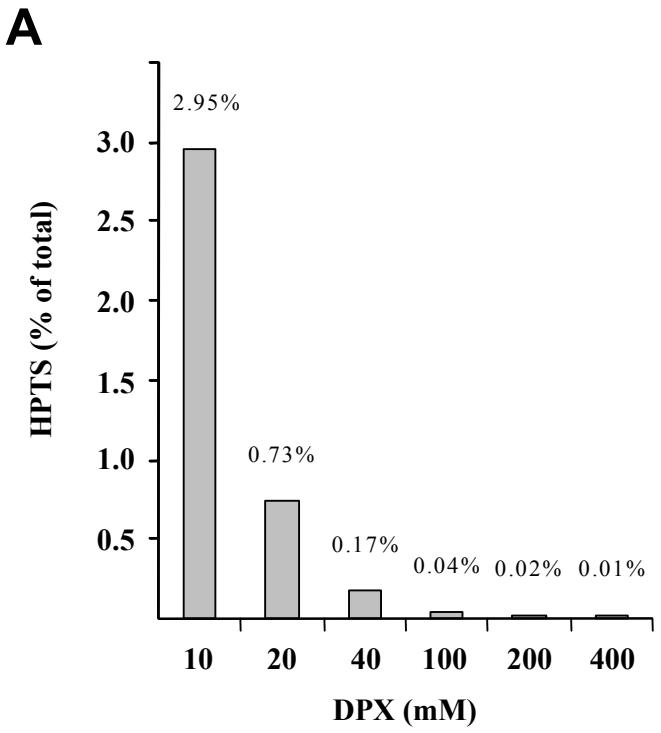
Figure S6. (A) Alix-Tsg101 interactions. (A) Alix-GST and GST were used in a pull-down assay from rat liver cytosol. $1/100^{th}$ of the input and the whole pull-down samples were

submitted to SDS-PAGE and immunoblotted against Alix and Tsg101 as indicated. **(B-C) Tsg101 knockdown in hamster cells. (B)** After a 3 day treatment with control or 3 different anti-Tsg101 siRNAs, PNSs were submitted to SDS-PAGE and western blot analysis against the indicated antigens. Short and long exposure times are represented. Note that anti-Tsg101 siRNA #2 treatment also induces Hrs down-expression. **(C)** Comparison of Tsg101 cDNA sequences between BHK and other indicated species. Anti-Tsg101 siRNA #1 target sequence is indicated in red. **(D) Effects of Alix or Hrs knockdown on HPTS incorporation into early endosomes.** The in vitro assay with protocol 1 (Figure 1A) was performed after Alix or Hrs knockdown as in Figure 6, C and D, but early endosomes were analyzed. Means (±SEM) of at least five independent experiments are shown**.**

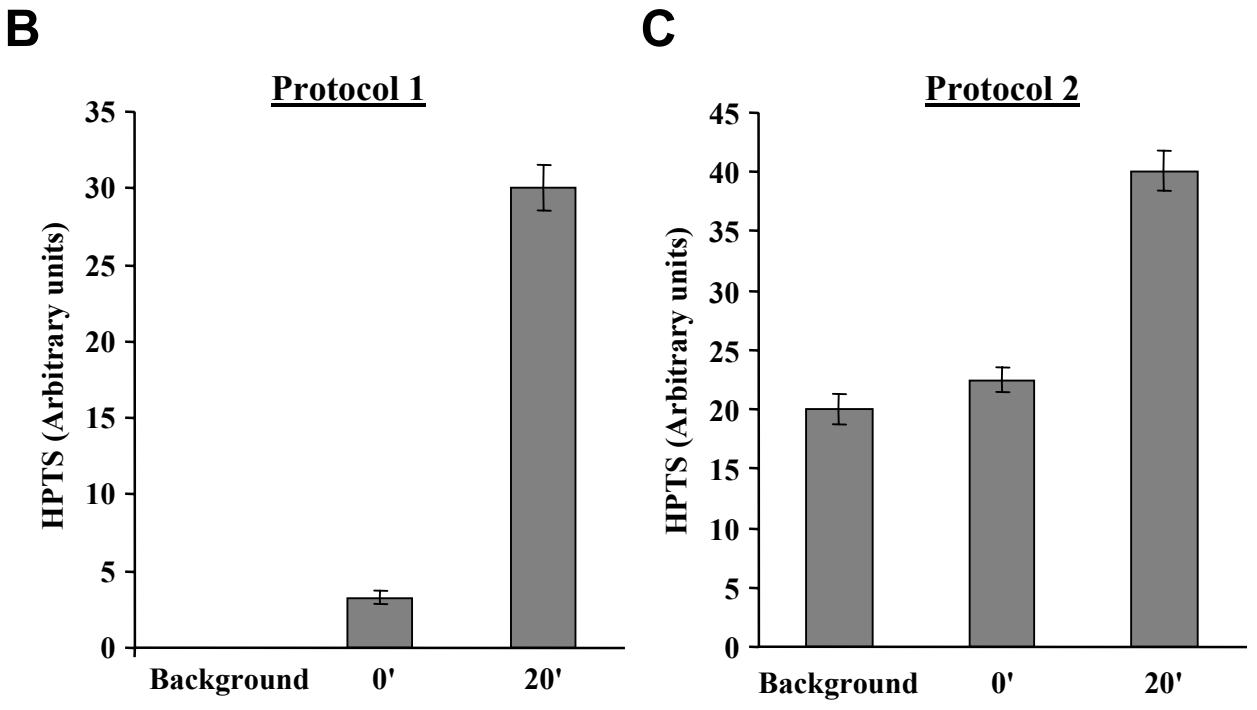
Figure S7. Analysis of hVps4 overexpression. (A) GFP, WT hVps4-GFP or hVps4K173Q-GFP were expressed for 16h in BHK cells, and then cells were fixed and analyzed by confocal microscopy. Bars: 20 μm. **(B)** PNSs from (A) were analyzed by western blotting with antibodies against the transferrin receptor (TfR) and GFP (left panels), as well as hVps4 (right panel). Note that the antibody does not cross-react with hamster Vps4. The position of GFP and the GFP-tagged versions of WT and mutant Vps4 are indicated by single and double starts, respectively. (A) and (B) are representative of three independent experiments.

Figure S8. Size of isolated late endosomes after Alix or Tsg101 knockdown. The perimeter of isolated late endosomes was measured using the NIH ImageJ Software for conditions shown in Figures 6 and 9 (mock-treated control, knockdown of Alix or Tsg101 and knockdown of both Alix and Tsg101). Means (±SEM) of 30 structures per condition from 3 independent experiments are represented.

HPTS quenching with DPX

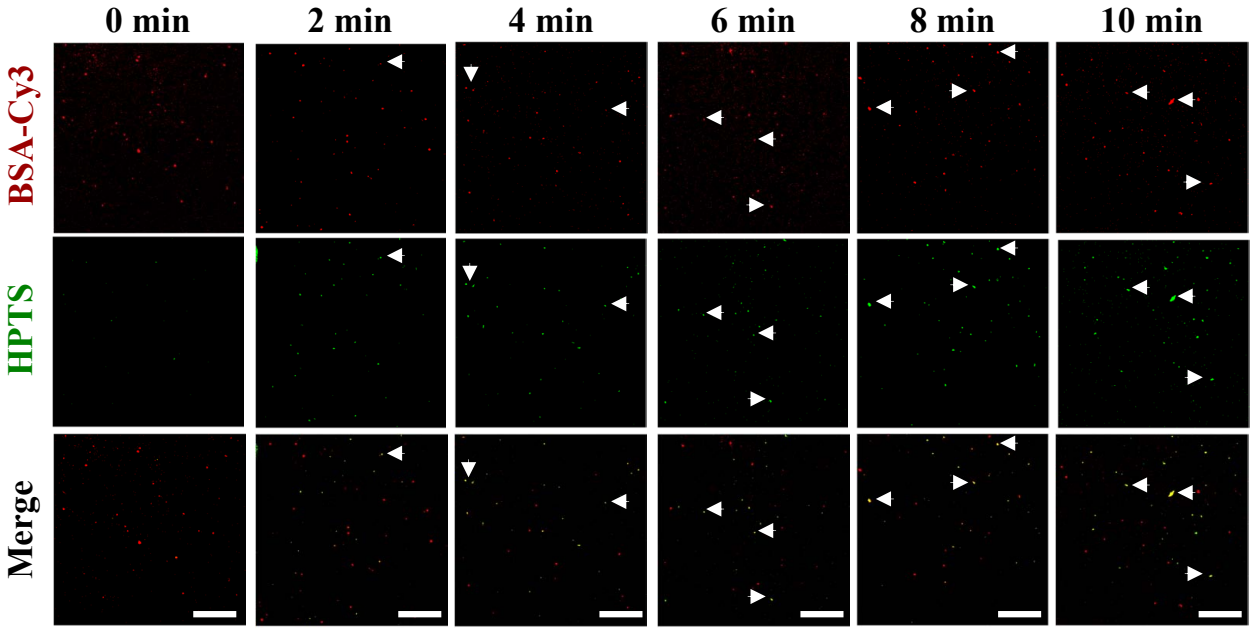


Background values of HPTS detection

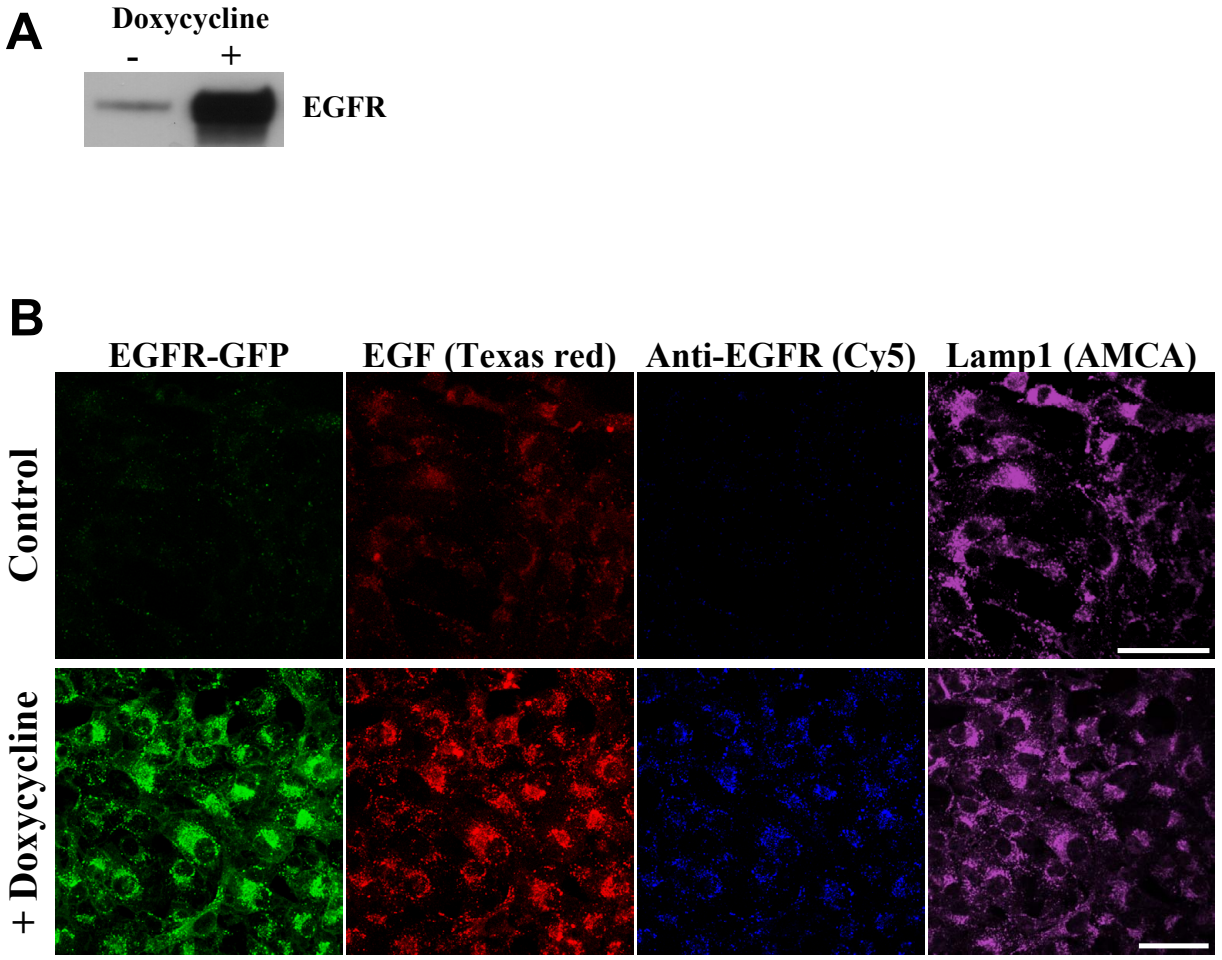


Falguières Fig S2

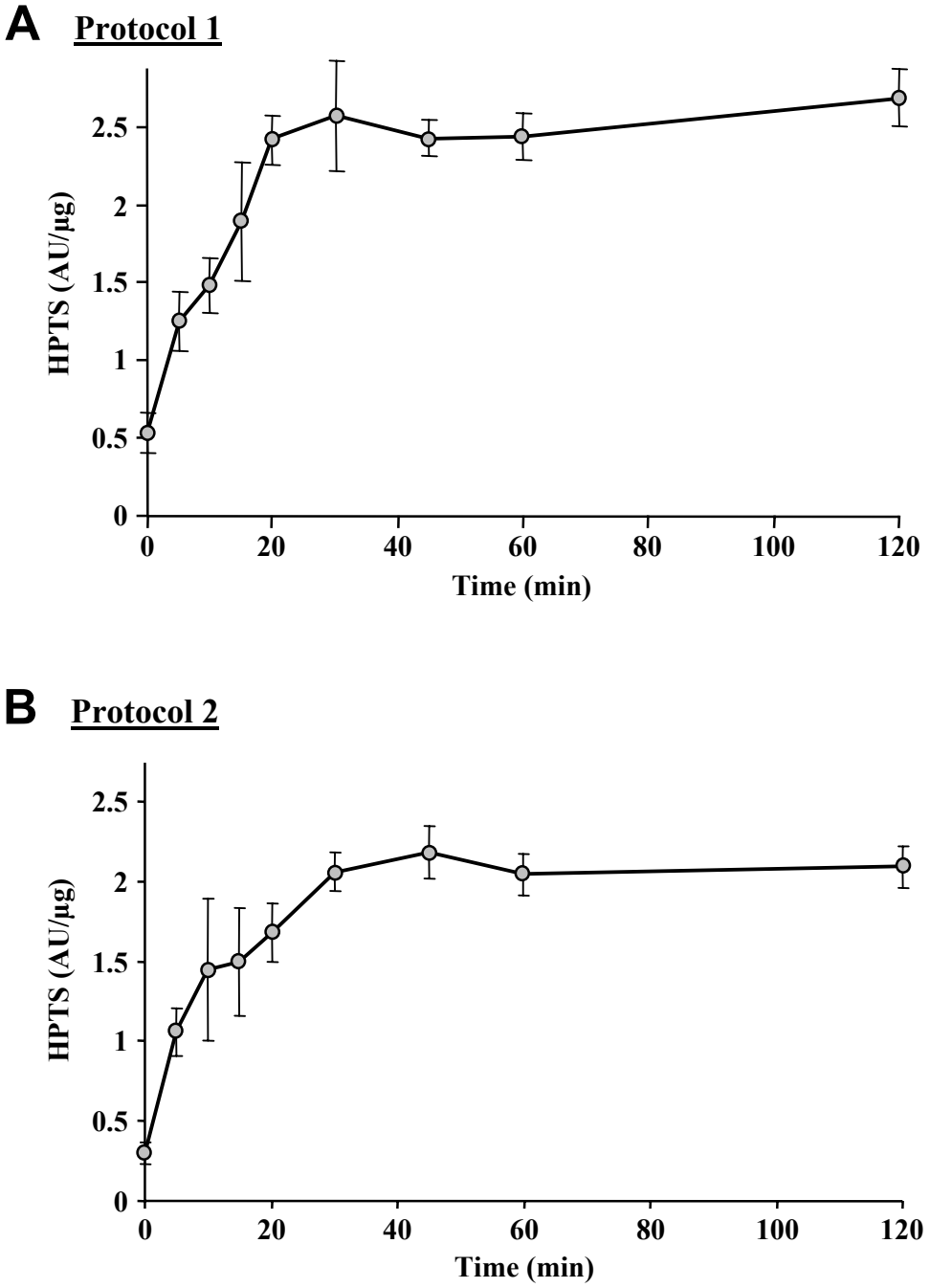
Time-course of HPTS accumulation in endosomes preloaded with BSA-Cy3



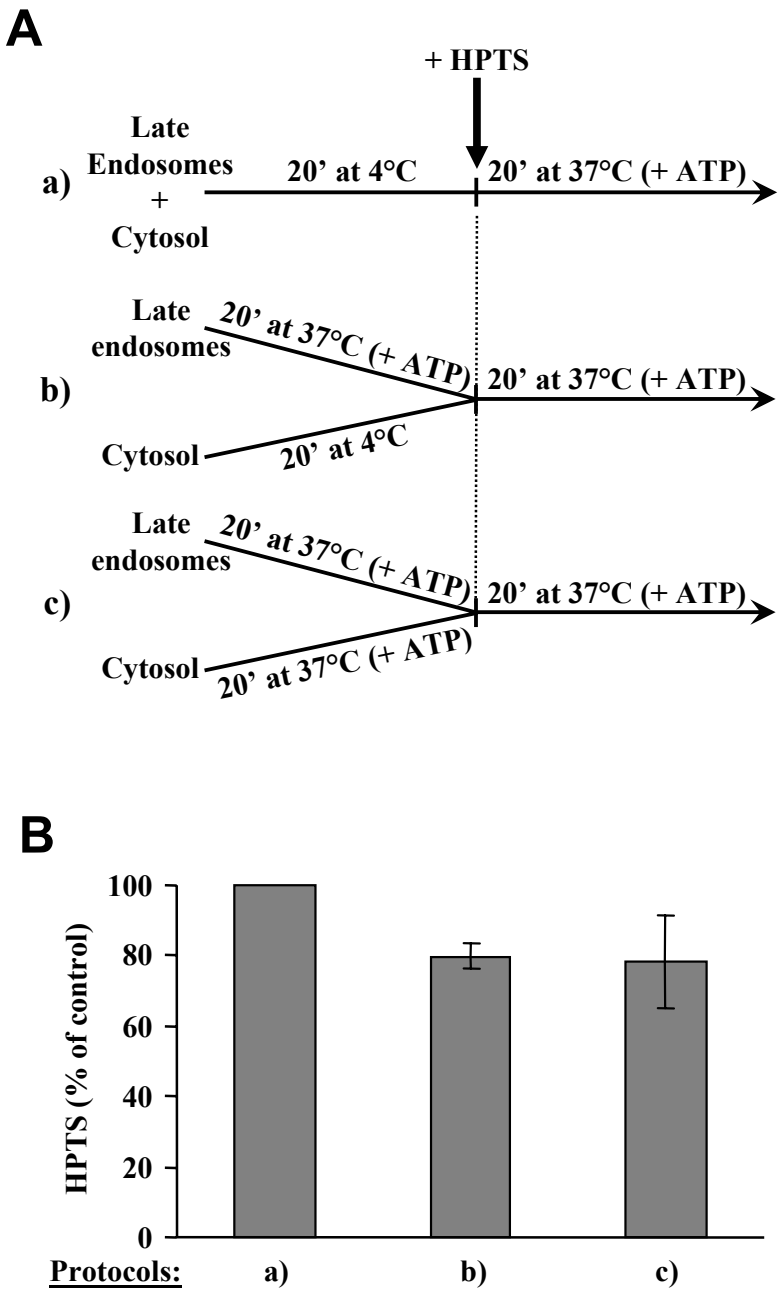
EGF and EGF receptor transport in BHK cells overexpressing the human EGF receptor



Time-course of HPTS incorporation

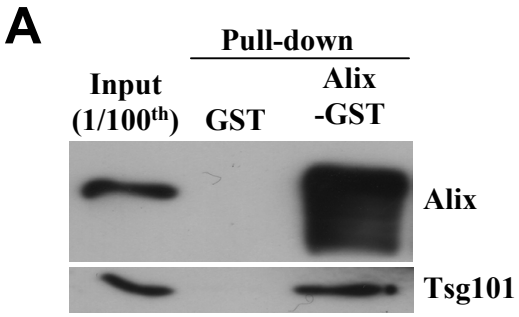


HPTS incorporation after endosome pre-incubation

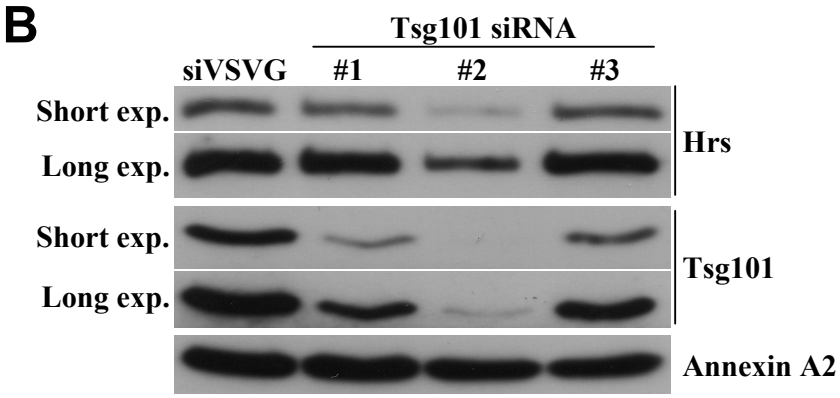


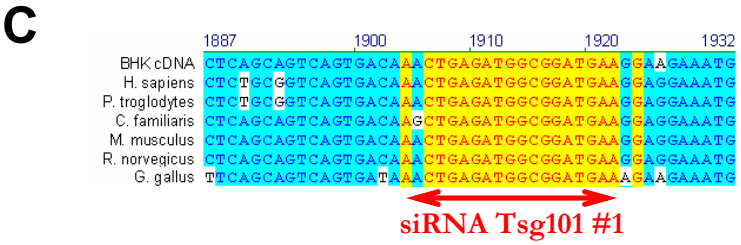
Falguières Fig S6

Alix-Tsg101 interactions

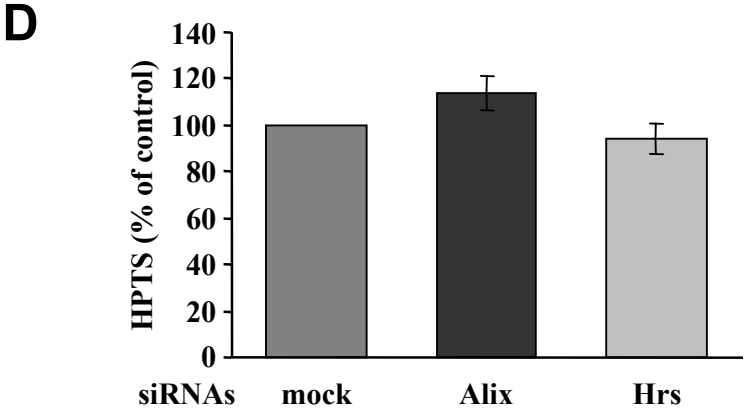


Tsg101 knockdowm in hamster cells

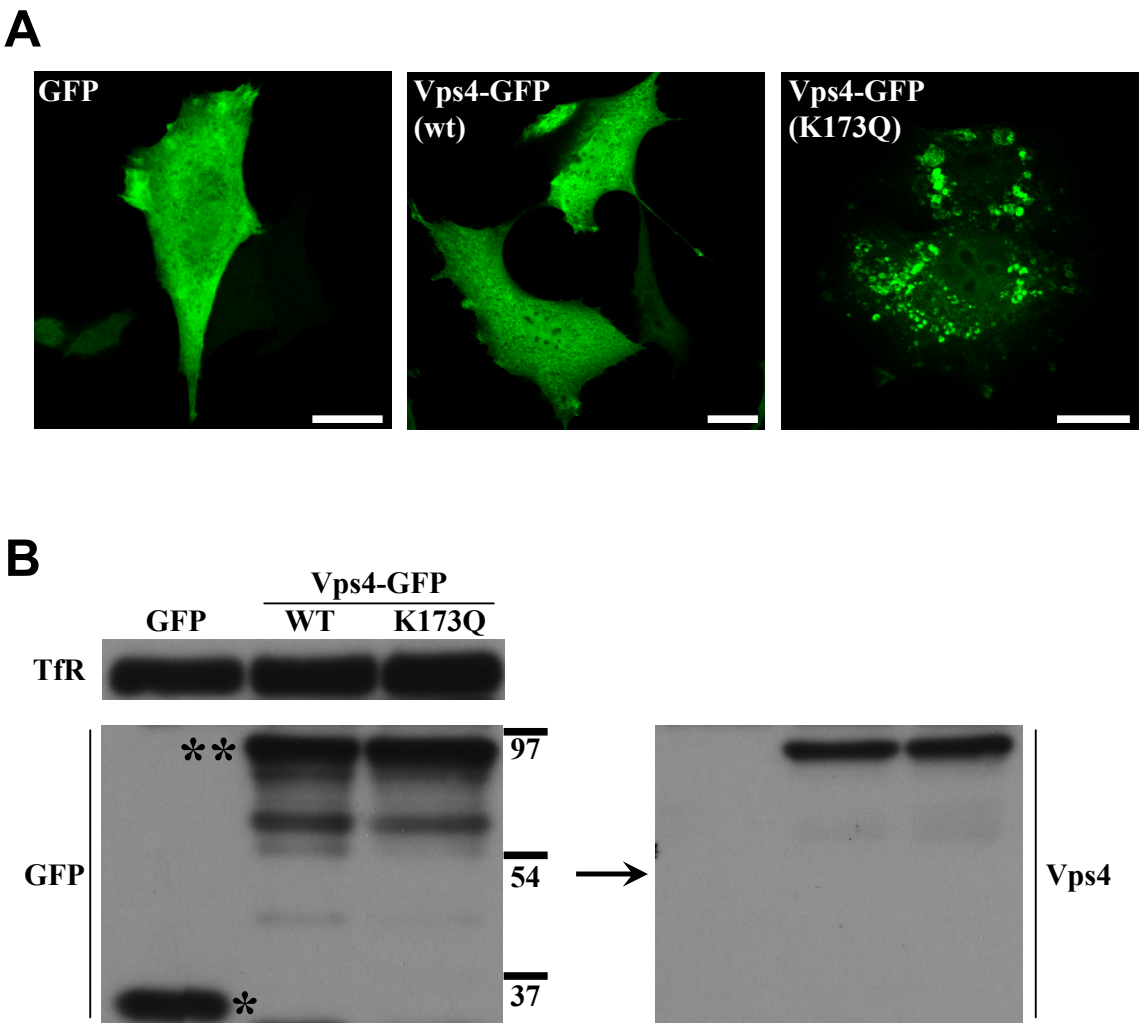




Effect of Alix and Hrs knockdown on early endosomes



hVps4 expression in BHK cells



Falguières Fig S8

Size of isolated late endosomes after knockdown of Alix or Tsg101