

Figure S1: Rab4a/b depletion studies. Related to Figure 2.

(A) Knockdown of Rab4a and Rab4b in Cos7 cells. Two pairs of sequences (Seq1 and Seq2) for Rab4a and Rab4b were used. Tubulin was used as loading control. (B) Quantitation of Rab4a/b knockdown using Seq1. (C) Cos7 cells were either mock (control) depleted or depleted of Rab4a/b, then fixed and stained for endogenous AP-1 (red) and EEA1 (blue). Arrows indicate no change in AP-1 staining at the TGN. Scale bar = 10 μ m.

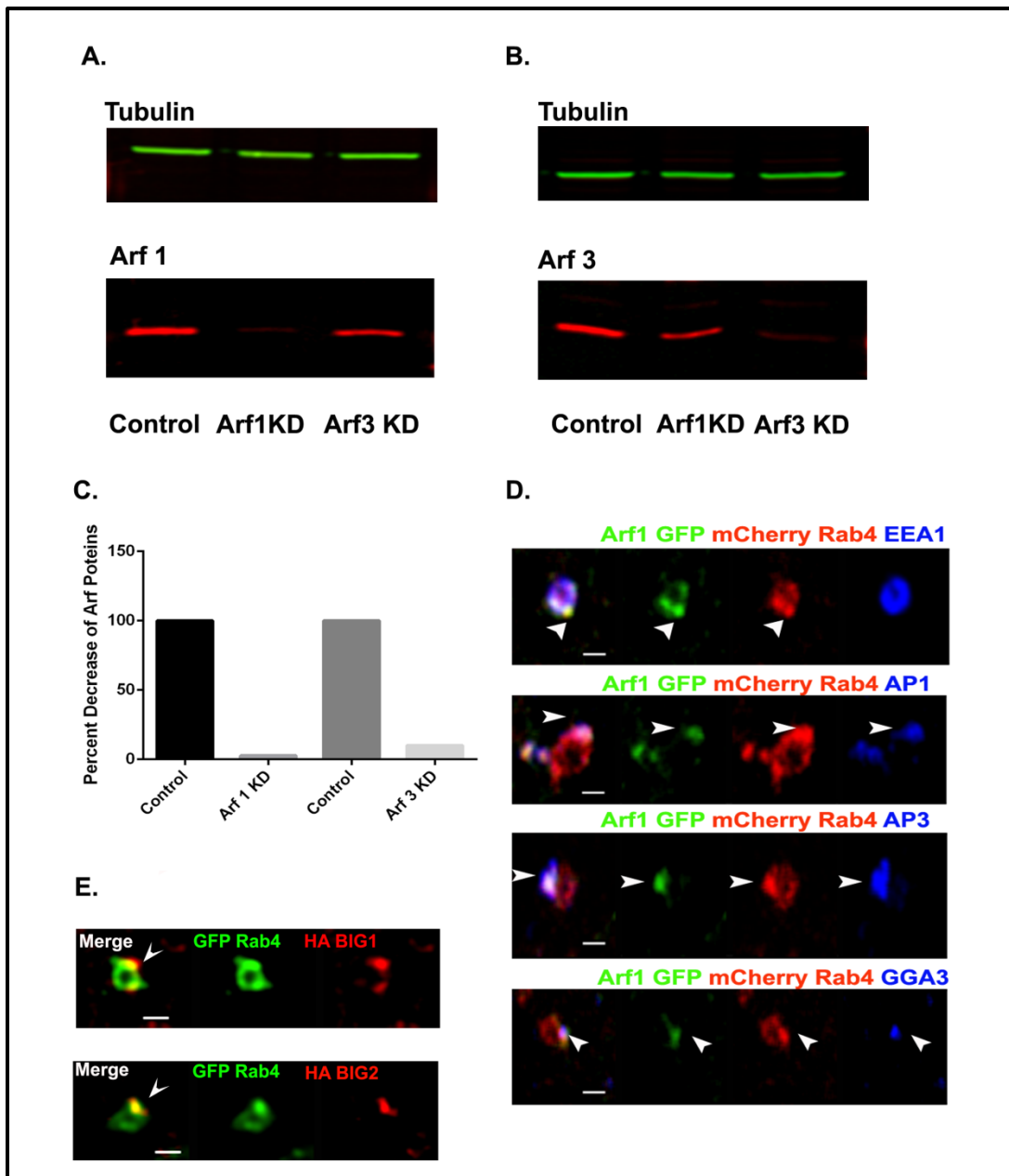


Figure S2: Arf 1 and Arf3 knockdown and localization of Class I Arfs and HA-BIG 1/2 to the Rab4 subdomain of the early endosome. Related to Figures 3 and 5.

(A) and (B) Cos7 cells were depleted of Arf1 or Arf3 individually and protein lysates were probed using anti-Arf1 or anti-Arf3 antibody. There was no change in Arf3 protein levels after Arf1 knockdown and vice versa, indicating the specificity of knockdown. Tubulin was used as loading control. (C) Quantitation of Arf1 and Arf3 knockdown. (D) Arf1 associates with the

Rab4 subdomain of the early endosome. The images shown are from Cos7 cells transfected with Arf1-GFP and mCherry-Rab4a, fixed and stained for endogenous EEA1 or adaptor proteins (AP-1, AP-3 and GGA-3). Arrows indicate colocalization of Arf1 (green) with the Rab4-positive bud (red) and not the EEA1-positive region (blue) of the endosome. The adaptor proteins also colocalize with Arf1 on the Rab4-positive buds of the endosome. Scale bar = 1 μ m.

(E) Localization HA tagged BIG1 and BIG2 on the Rab4 subdomain. Cos7 cells were transfected with HA tagged BIG1/BIG2 and GFP-Rab4a, fixed and stained using anti-HA antibodies. Arrowheads show the localization of BIGs (red) on the Rab4-positive buds (green). Scale bar = 1 μ m.

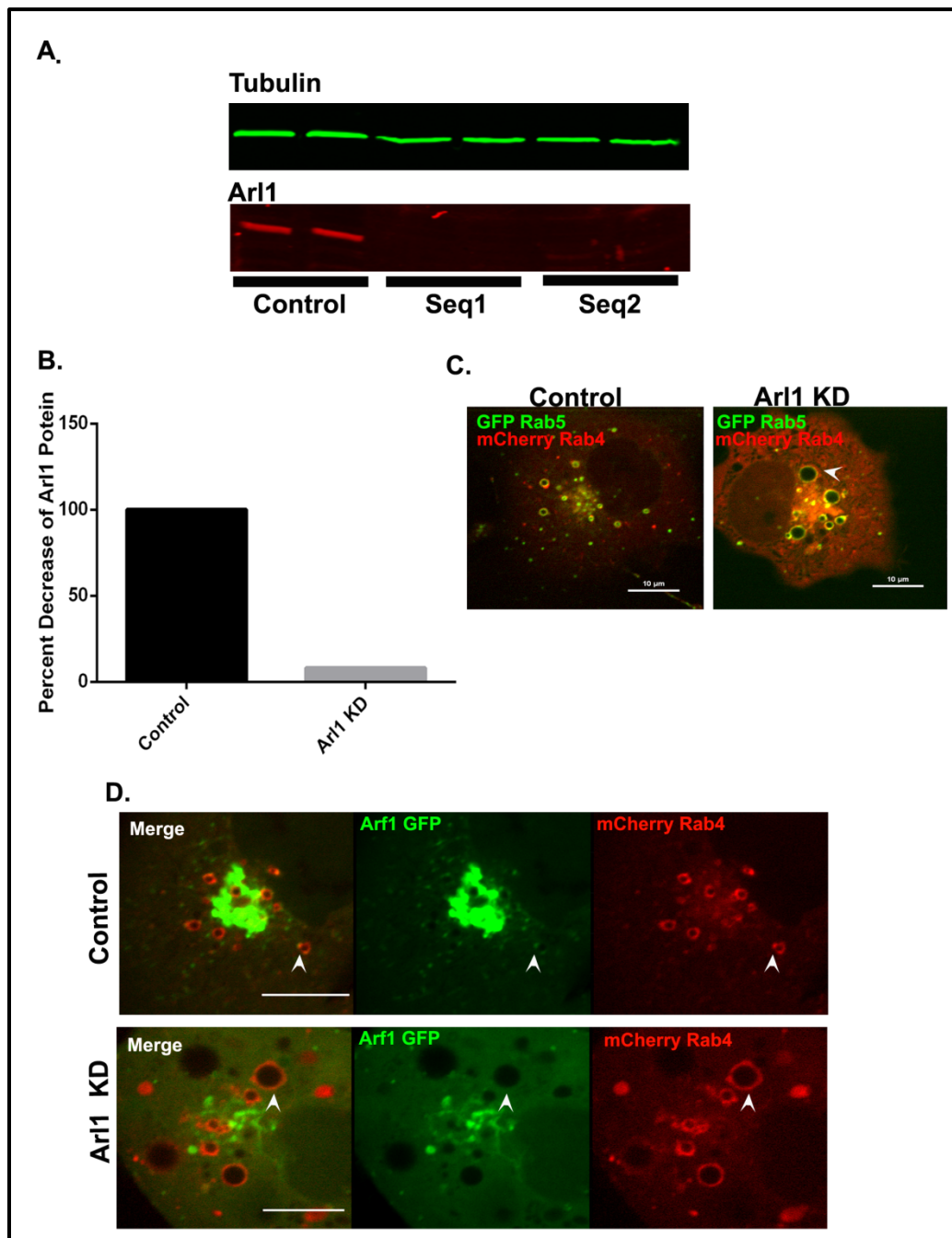


Figure S3: Arl1 depletion studies. Related to Figures 6 and 7.

(A) Cos7 cells were depleted of Arl1. Two sequences (Seq1 and Seq2) were used and tubulin was used as loading control. (B) Quantitation of Arl1 knockdown using Seq1. (C) Still images from live cell movies of control and Arl1 depleted Cos7 cells expressing GFP-Rab5 and

mCherry-Rab4a. Arrowheads indicate the presence of Rab5 (green) on the enlarged Rab4-positive endosomes (red) after Arl1 depletion. Scale on cells = 10 μ m. **(D)** Still images from movies of control and Arl1 depleted Cos7 cells expressing Arf1-GFP and mCherry-Rab4a. Arrowheads indicate the presence or absence of Arf1 (green) on Rab4-positive buds (red). Scale on cells = 10 μ m.

Legends for Supplemental Movies

Movie S1: Rab4-positive buds separate from Rab5 endosomes. Related to Figure 1.

This movie shows the emergence of Rab4-enriched buds (red) from Rab5-positive endosomes (green). Images were captured at a rate of 1 frame per sec for 10sec. Scale bar = 1 μ m.

Movie S2: Arf1 is present on Rab4-positive buds. Related to Figure 3.

This movie shows the concentration of Arf1 (green) on Rab4 (red) positive buds emerging from endosomes. Images were captured at a rate of 1 frame per sec for 20 sec. Scale bar = 1 μ m.

Movie S3: Cos7 cell expressing Arf1-mCherry and GFP-Rab4a. Related to Figure 4.

A 7.5 minute movie of Cos7 cells, imaged before BFA treatment showing Arf1 (red) association with Rab4-positive buds (green). Images were captured at a rate of 1 frame per 3 seconds. Scale bar = 10 μ m.

Movie S4: BFA treatment results in loss of Arf1 from Rab4 positive buds. Related to Figure 4.

This movie shows dissociation of Arf1 (red) and tubulation of Rab4-positive endosomes after BFA treatment. BFA was added at the start of the movie and imaged at a rate of 1 frame per 3 seconds for 7.1 mins. Scale bar = 10 μ m.

Movie S5: BFA treatment results in tubulation of Rab4 positive buds. Related to Figure 7.

This movie shows tubulation of Rab4-positive (green) endosomes in control cells after BFA treatment. Images were captured immediately after the addition of BFA at a rate of 1 frame per 3 seconds for 10 mins. Scale bar =10 μ m.

Movie S6: BFA treatment does not affect Rab4 endosomes in Arl1 depleted cells. Related to Figure 7.

Cos7 cells depleted of Arl1, treated with BFA and imaged at a rate of 1 frame per 3 seconds for 10 minutes. No tubulation of Rab4-positive (green) endosomes was seen in the presence of BFA. Scale bar =10 μ m.