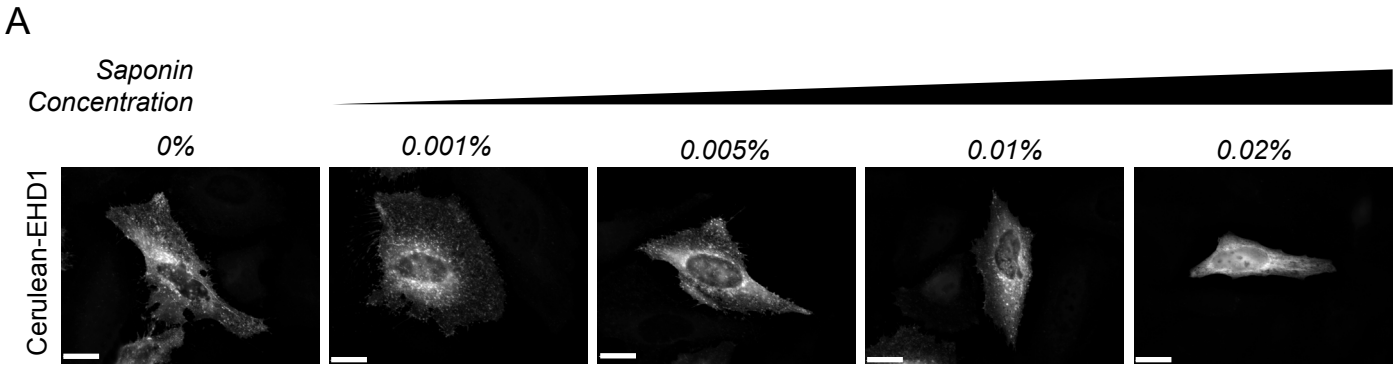
Supplemental Materials

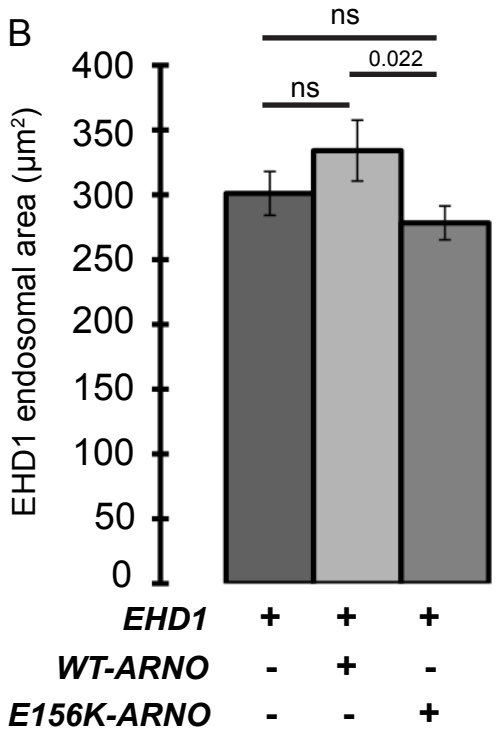
Molecular Biology of the Cell

Salem et al.

Supplemental Table 1: Pearson's Correlation Coefficients		
	0% Saponin	0.02% Saponin
WT-ARNO/EHD1	0.72 (n = 40 cells)	0.74 (n = 64 cells)
E156K-ARNO/ EHD1	0.67 (n = 75 cells)	0.80 (n = 50 cells)

Supplemental Table 1: Pearson's Correlation coefficients for cytohesin-2/ARNO and EHD1. Cerulean-EHD1 channels were masked, and co-localization with cytohesin-2/ARNO (WT or E156K) were measured for cells treated with 0% and 0.02% saponin permeabilization buffer. Co-localization was quantified using 2 independent experiments with the following number of cells: (0% saponin EHD1/WT-ARNO; n = 40 cells, 0% saponin EHD1/E156K-ARNO; n = 75 cells; 0.02% saponin EHD1/WT-ARNO; n = 64 cells, 0.02% saponin EHD1/E156K-ARNO; n = 50 cells).

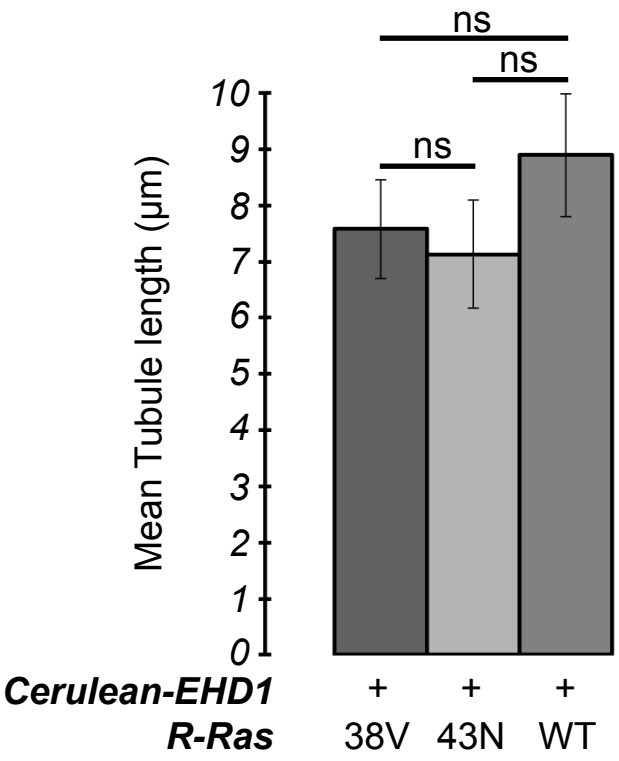




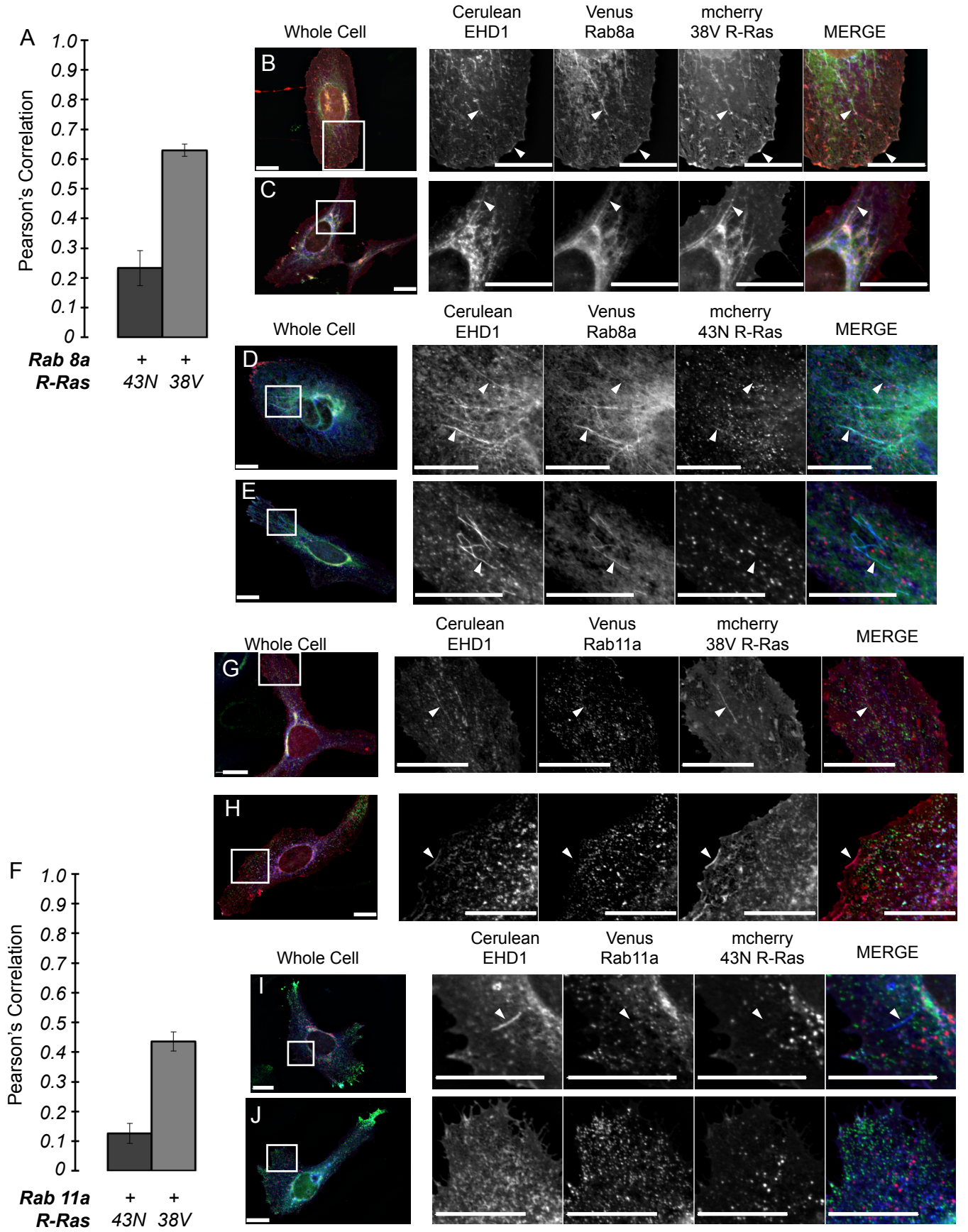
Supplemental Figure 1: Saponin treatment does not promote EHD1 tubule formation. A) HeLa cells expressing Cerulean-EHD1 were treated with saponin permeabilization buffer from 0% - 0.02% saponin. Cells were imaged using wide field microscopy, and deconvolved using Slidebook 6.0 imaging software. Images were taken over the course of 3 independent experiments. B) A 2D-Laplacian filter was applied to EHD1 channels and subsequently masked to highlight EHD1-positive structures. Raw endosomal area for 0% saponin-treated cells (Figure 1 A-C) was extracted using Slidebook 6.0 software. Samples were analyzed from 2 independent experiments using a 2-sample t-test using MiniTab 17. ns = not significant.

Supplemental Table 2: Pearson's Correlation Coefficients		
Cerulean-EHD1 mcherry WT R-Ras	Cerulean-EHD1 mcherry 38V R-Ras	Cerulean-EHD1 mcherry 43N R-Ras
0.51 (n = 60 cells)	0.54 (n = 65 cells)	0.56 (n = 51 cells)

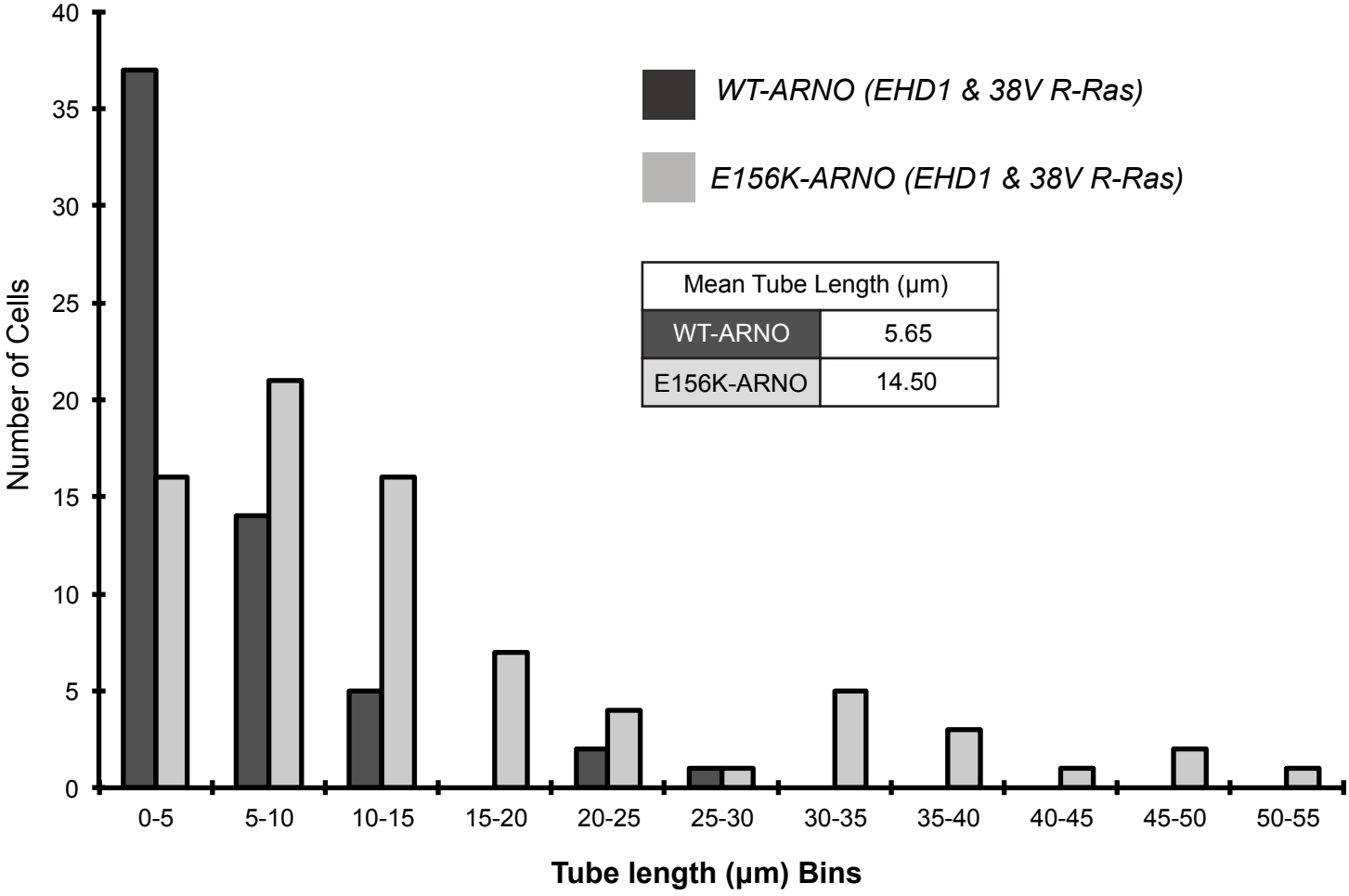
Supplemental Table 2: Wild-type and mutant R-Ras (38V and 43N) share similar co-localization values. Images were deconvolved and R-Ras channels were masked. Pearson's Correlation was determined using Slidebook 6.0 software. (38V R-Ras; n = 65 cells, 43N R-Ras; n = 51 cells, WT R-Ras, n = 60 cells).



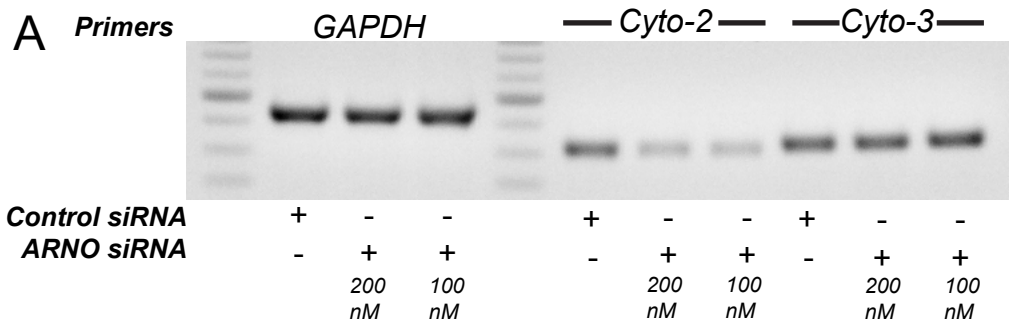
Supplemental Figure 2: R-Ras does not affect EHD1 tubule length. The longest EHD1 endosome per cell was measured in cells expressing Cerulean-EHD1 and mcherry tagged versions of mutant R-Ras (38V or 43N) or WT R-Ras (Figure 2). Samples were analyzed using a 2-sample t-test using MiniTab 17. ns = not significant.

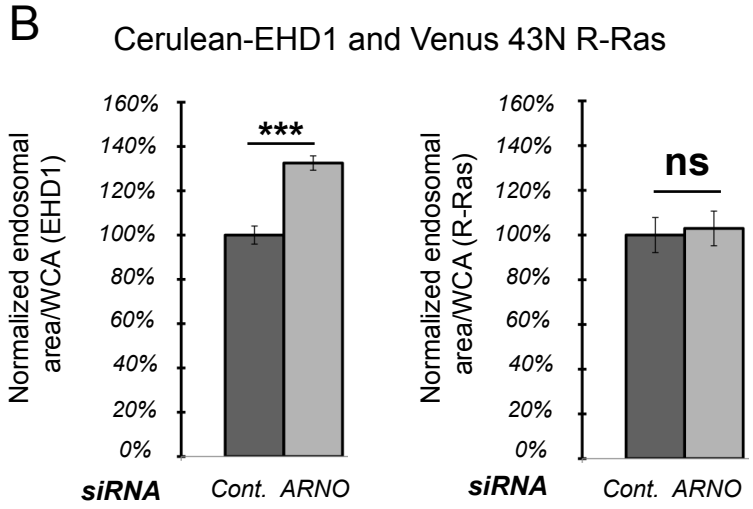


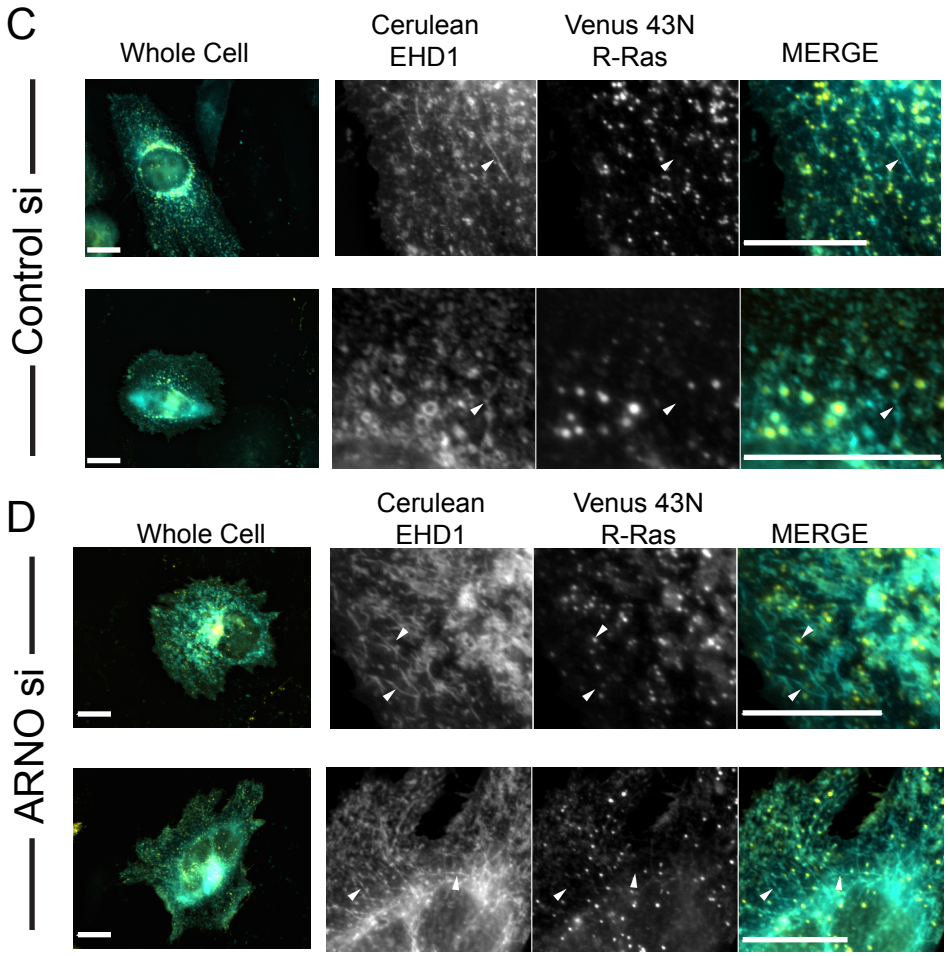
Supplemental Figure 3: 38V R-Ras shows higher co-localization with Rab8a than Rab11a. R-Ras channels were masked, and the Pearson's Correlation coefficients were exported from Slidebook 6.0. Rab8a showed co-localization with 38V R-Ras (Pearson's = 0.63) at tubular EHD1 endosomes and at the peripheral plasma membrane (see white arrows). Rab11a and 38V R-Ras showed partial co-localization (Pearson's = 0.44) but, Rab11a was noticeably absent from the plasma membrane and EHD1 tubular endosomes in most cells. 43N R-Ras did not co-localize with either Rab8a (Pearson's = 0.21) or Rab11a (Pearson's = 0.11). EHD1 is pseudocolored blue, Rabs are green, and R-Ras is red.



Supplemental Figure 4: Distribution of tubule length in cells expressing WT-ARNO vs. E156K-ARNO. The longest tubule in each cell from the experiment presented in Figure 3 was measured using Slidebook 6.0 software. Cells with E156K-ARNO have an average tube length (mean = 14.50 μm) about 2.5 times longer than WT-ARNO cells (mean = 5.646 μm). Measurements were obtained from a total of 136 cells.





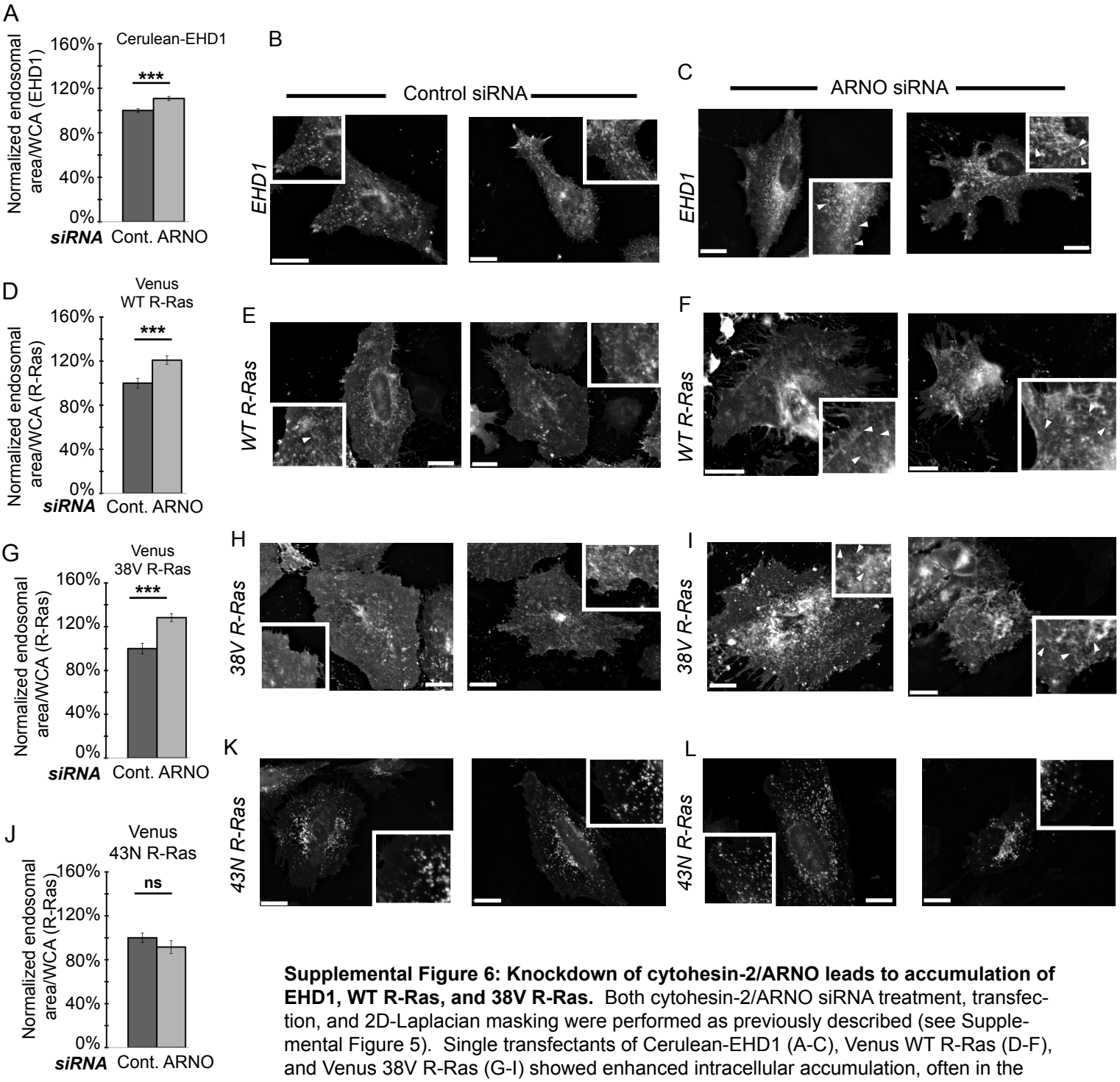


Supplemental Figure 5: Cytohesin-2/ARNO knockdown does not change intracellular accumulation of 43N R-Ras. (A) One Step RT-PCR (Qiagen) using GAPDH primers as a control, primers targeting the PH-domain of human cytohesin-2/ARNO, and cytohesin-3/GRP1. Two ARNO knockdowns using 200 nM and 100 nM siRNA were performed. Band intensity of gel images were analyzed in ImageJ. A ~75% knockdown of ARNO was achieved for both knockdowns. (B) Cytohesin-2/ARNO knockdown promotes intracellular accumulation of EHD1 but not 43N R-Ras. (C) Control siRNA treated cells show EHD1 tubular endosomes devoid of 43N R-Ras, but maintain EHD1-doughnut shaped structures filled with R-Ras. (D) While ARNO siRNA treated cells show highly tubular endosomes and intracellular levels of EHD1, 43N R-Ras morphology is unchanged. EHD1 is pseudocolored cyan and R-Ras is yellow. The following number of cells from one experiment were analyzed: (EHD1/43N R-Ras control and ARNOsi; n = 118 cells). ***p < 0.001, ns = not significant. Scale bars = 20 μm.

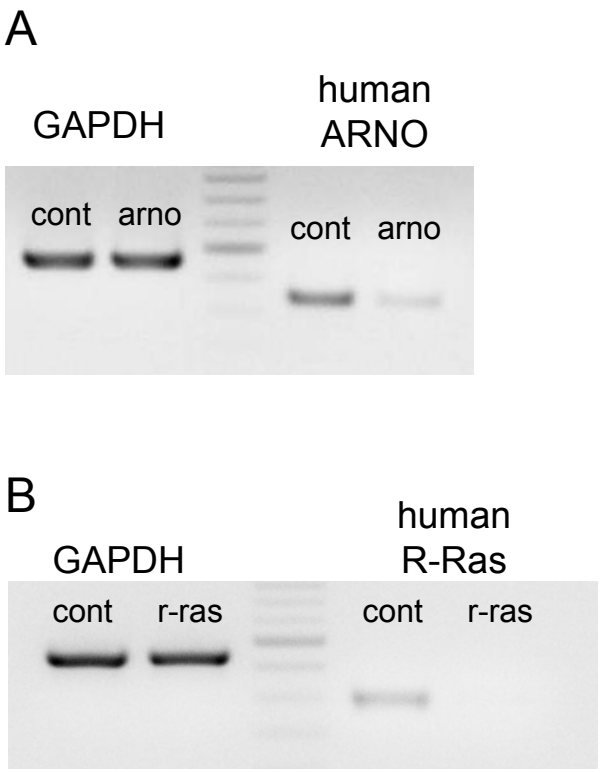
Supplemental Table 3A: Percentage of EHD1 Objects (endosomes) within a given range								
PERIMETER	EHD1 (Cont)	EHD1 (ARNOsi)	EHD1/WT (Cont)	EHD1/WT (ARNOsi)	EHD1/38V (Cont)	EHD1/38V (ARNOsi)	EHD1/43N (Cont)	EHD1/43N (ARNOsi)
% < 5 µm	28.64%	28.80%	25.74%	25.40%	34.78%	26.97%	32.25%	28.19%
% 5-10 µm	49.37%	46.01%	47.18%	45.78%	49.52%	50.11%	49.92%	48.65%
% >10 µm	21.99%	25.20%	27.08%	28.82%	15.70%	22.92%	17.83%	23.16%
MAJOR AXIS LENGTH	EHD1 (Cont)	EHD1 (ARNOsi)	EHD1/WT (Cont)	EHD1/WT (ARNOsi)	EHD1/38V (Cont)	EHD1/38V (ARNOsi)	EHD1/43N (Cont)	EHD1/43N (ARNOsi)
% < 2 µm	9.20%	12.22%	8.45%	8.55%	10.40%	6.83%	9.69%	7.68%
% 2-4 µm	40.43%	36.86%	39.42%	38.03%	45.92%	38.92%	43.83%	40.93%
% 4-6 µm	24.94%	23.70%	24.16%	23.68%	24.03%	26.58%	24.58%	24.88%
% 6-8 µm	11.99%	11.46%	11.70%	11.62%	9.46%	12.04%	10.90%	11.85%
% > 8 µm	13.44%	15.76%	16.27%	18.12%	10.19%	15.64%	11.00%	14.66%

Supplemental Table 3B: Mean Morphometry Values (µm)								
	EHD1 (Cont)	EHD1 (ARNOsi)	EHD1/WT (Cont)	EHD1/WT (ARNOsi)	EHD1/38V (Cont)	EHD1/38V (ARNOsi)	EHD1/43N (Cont)	EHD1/43N (ARNOsi)
Mean PERIMETER (µm)	8.58	9.57	9.83	10.33	7.50	8.96	7.72	8.76
Mean MAJOR AXIS LENGTH (µm)	1.76	1.81	1.85	1.91	1.64	1.84	1.68	1.80

Supplemental Table 3: Cytohesin-2/ARNO knockdown results in more EHD1 endosomes with large perimeter and major axis length. (A) EHD1 2D-Laplacian filter masks were exported as individual objects from Slidebook 6.0 in control and ARNOsi cells. Objects within a given range were counted and calculated as the percentage of total objects. (B) Raw perimeter (µm) and major axis length (µm) values were averaged. Green boxes highlight increases in ARNO siRNA treated cells.



Supplemental Figure 6: Knockdown of cytohesin-2/ARNO leads to accumulation of EHD1, WT R-Ras, and 38V R-Ras. Both cytohesin-2/ARNO siRNA treatment, transfection, and 2D-Laplacian masking were performed as previously described (see Supplemental Figure 5). Single transfectants of Cerulean-EHD1 (A-C), Venus WT R-Ras (D-F), and Venus 38V R-Ras (G-I) showed enhanced intracellular accumulation, often in the form of more tubule formation in cells where cytohesin-2/ARNO levels were reduced (white arrows). Venus 43N R-Ras (J-L) did not show any tube formation or change in accumulation. The following number of cells from one experiment were analyzed: (EHD1; n = 239 cells, WT R-Ras; n = 176 cells, 38V R-Ras; n = 106 cells, 43N R-Ras; 106 cells). ***$p < 0.001$, ns = not significant. Scale bars = 20 μm.



Supplemental Figure 7: One Step RT-PCR (Qiagen) using GAPDH primers as a control, and primers targeting the PH-domain of human cytohesin-2/ARNO (A) or R-Ras (B). Universal control siRNA was used as a control. Band intensity of gel images were analyzed in ImageJ. A 80% knockdown of ARNO was achieved and over 90% of R-Ras knockdown was achieved for cells that were analyzed for cell spreading (B).