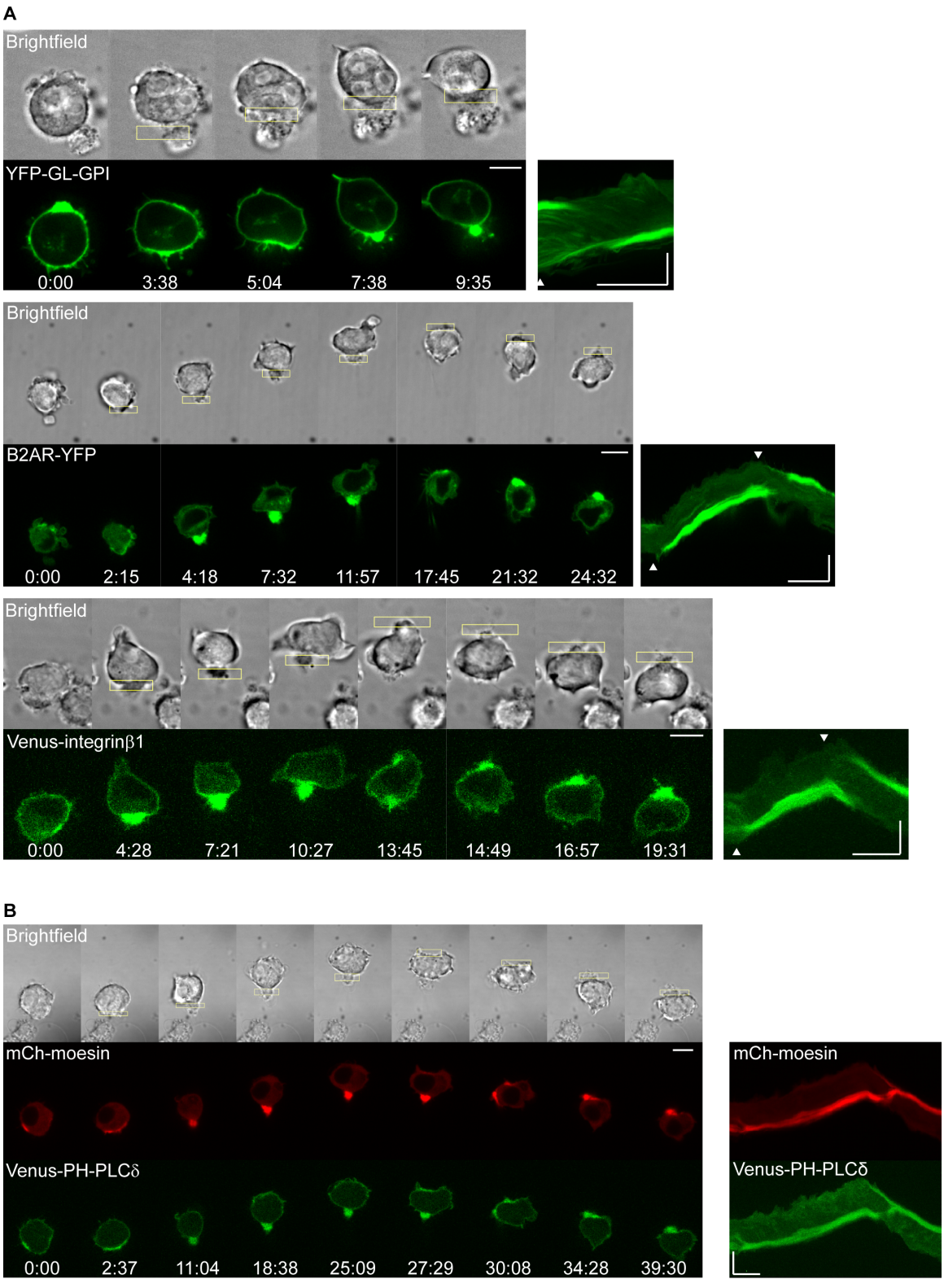
A
Brightfield
YFP-GL-GPI
0:00
3:38
5:04
7:38
9:35
Brightfield
B2AR-YFP
0:00
2:15
4:18
7:32
11:57
17:45
21:32
24:32
Brightfield
Venus-integrinβ1
0:00
4:28
7:21
10:27
13:45
14:49
16:57
19:31
B
Brightfield
mCh-moesin
Venus-PH-PLCδ
0:00
2:37
11:04
18:38
25:09
27:29
30:08
34:28
39:30
mCh-moesin
Venus-PH-PLCδ

Figure S1, related to Figures 3 and 5.

(A) Rearward flow of plasma membrane markers. Optically driven migration of a cell expressing LARG-mCh-SspB, iLID-CaaX, and either YFP-GL-GPI (top, green), β2AR-YFP (middle, green), or Venus-integrinβ1 (bottom, green).

(B) Moesin and PIP2 distribution during RhoA-driven migration. Optically driven migration of a cell expressing LARG-mTurq-SspB, iLID-CaaX, mCh-moesin (red) and the PI(4,5)P2 sensor Venus-PH-PLCδ (green). Kymographs are shown on the right. Yellow rectangles in the brightfield images mark the sites of photoactivation. Image sequence scale bars are 10 μm. Kymograph scale bars are 10 μm (vertical) and 5 min (horizontal). Time is in min:sec.

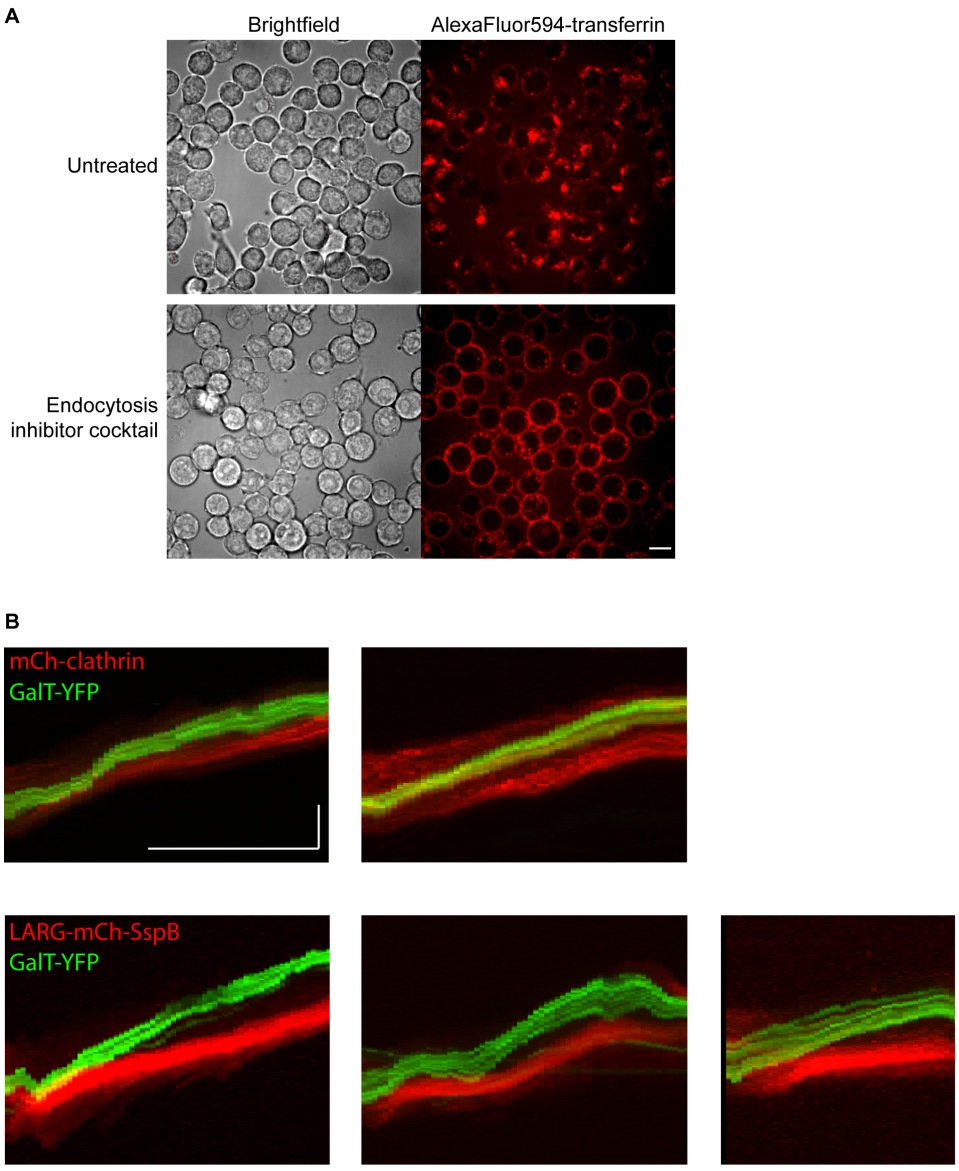

Figure S2, related to Figure 4.

(A) Effects of endocytosis inhibitor cocktail on transferrin internalization. Prior to transferrin addition, untransfected cells were treated for 30 min with vehicle (0.41% DMSO) or endocytosis inhibitor cocktail (10 μM secinH3, 25 μM pitstop2, 100 μM dynasore). Images were acquired 5 min after adding transferrin. Scale bar is 10μm.

(B) Golgi localization during RhoA driven migration. Kymographs showing the localization of the Golgi marker GalT-YFP (green) over time during RhoA driven cell

migration. The Golgi moves toward the front of the cell, whereas clathrin and the RhoA GEF construct (red) can be seen localized to the rear.

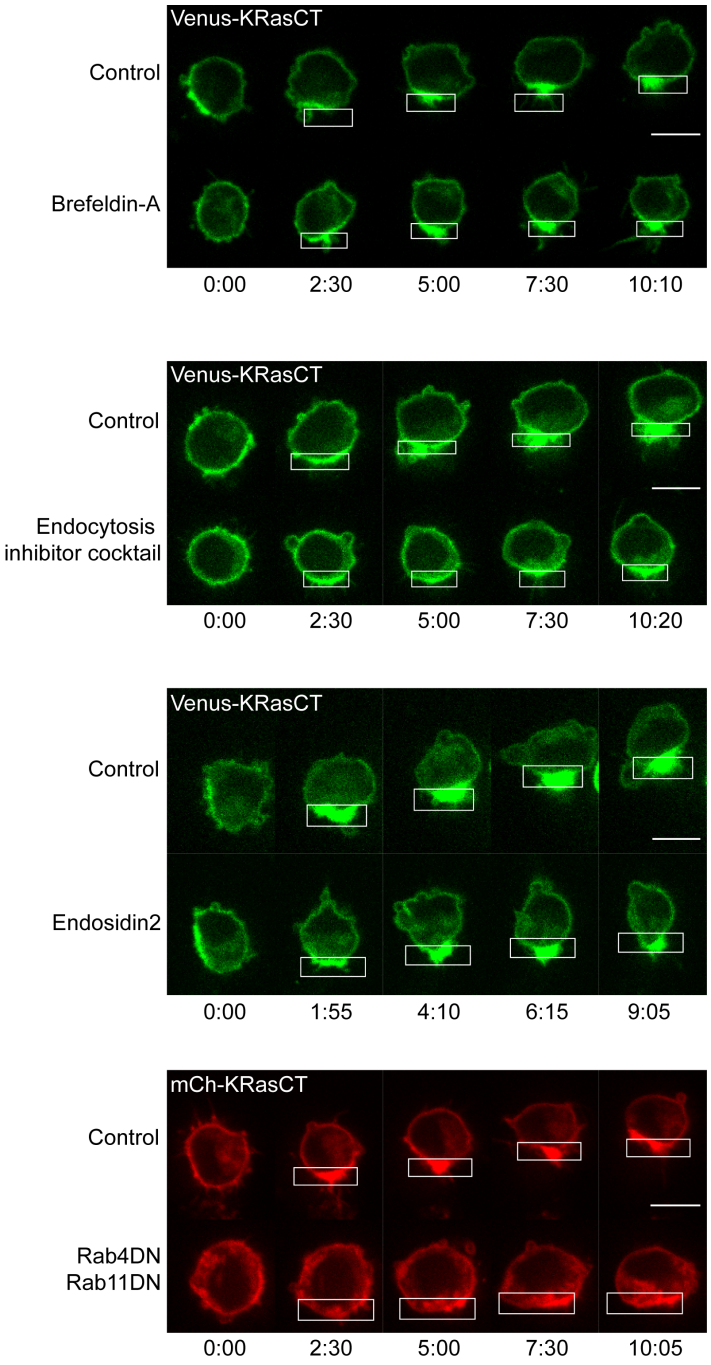

Figure S3, related to Figure 4.

Pharmacological and genetic perturbations of membrane trafficking and RhoA driven migration. Images show representative cells for the plots in Figure 4.

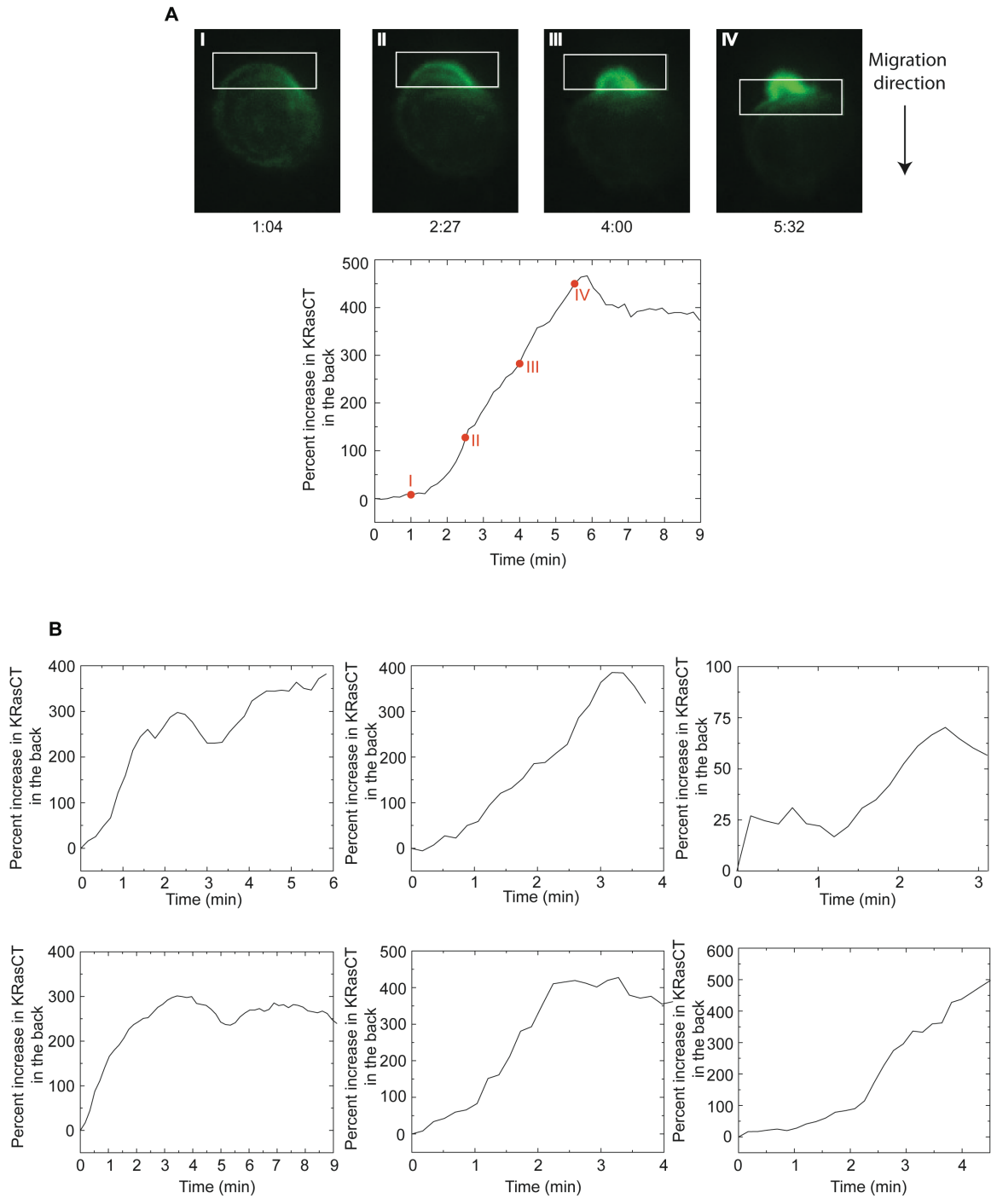

Figure S4, related to Figure 6.

KrasCT accumulation at the cell rear during RhoA driven migration of suspended cells. (A) Image sequence shows RhoA driven migration in a suspended cell expressing

the Venus-KRasCT. Images are sum intensity projections from 3D z-stack image data. White rectangles mark the photoactivated sites. The plot shows the change in fluorescence intensity within a 2 x 5 μm region at the cell rear over time. Individual time points corresponding to the images are marked. (B) Plots for 6 additional cells undergoing RhoA driven migration in suspension.

Gene Name	Expression (reads per kilobase per million reads)
RhoA	194
RhoB	23
RhoC	85
Rac1	131
Rac2	146
Rac3	0.3

Table S1, related to Figures 1 and 2. Expression levels of Rho and Rac isoforms.

RNAseq data for RAW 264.7 cells.